Studying the breeding ecology of *Culicoides* (Diptera: Ceratopogonidae) with focus on the Obsoletus Group

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Contents

CHAPTER 1

GENERAL INTRODUCTION

The Ceratopogonidae are a very diverse and widespread family of Diptera, commonly referred to as "biting midges", although the species of only four genera actually feed on the blood of vertebrates. Over 6,000 described species are placed in 103 genera. Of most noticeable medical and veterinary relevance are the species of the genus *Culicoides* (Figure 1.1). With only one to three mm in size, they are among the smallest haematophagous insects. About 1,400 *Culicoides* species have been described to date and with only few exceptions, e.g., the Polar Regions or New Zealand, they occur worldwide (Blackwell, 2008; Borkent, 2015). In many regions, species of the genus *Culicoides* are known as pests to humans. In Western Scotland and parts of England, the nuisance species *C. impunctatus* Goetghebuer adversely affects local tourism (Blackwell and Page, 2003). The bites of *C. paraensis* (Goeldi), a vector of the Oropouche virus in the Neotropical region, cause skin problems and allergies (Tesh, 1994; Felippe-Bauer and Sternheim, 2008). A common physiological reaction of men to the saliva of the biting female is the characteristic itching and swelling of the wound. Also severe allergic reactions are possible, but are rarely reported in humans (reviewed by Hoffman, 1987).

More crucial than the biting nuisance, is the role of *Culicoides* in transmission of pathogens with veterinary relevance. In northern Europe, *Culicoides* became a sudden threat with the outbreak and rapid spread of serotype 8 of the bluetongue virus (BTV) in 2006 and 2007. Prior occurrences were mainly restricted to the distributional limits of *C. imicola* Kieffer, the principal vector of BTV in Africa and the Mediterranean regions (reviewed by Mellor et al., 2000; Meiswinkel et al., 2004b; Wilson and Mellor, 2009). Therefore, the unexpected BTV epidemic motivated extensive entomological surveys in the affected countries that resulted in two main conclusions: *C. imicola* was still absent from northern and central Europe, whereas the species of the Obsoletus Group were widely distributed. Moreover, being tested positive for BTV-8 strongly suggested that the species responsible for virus transmission belong to this group (Mehlhorn et al., 2007; Meiswinkel et al., 2008), comprising *C. obsoletus* sensu stricto (s.s.) (Meigen), *C. scoticus* Downes and Kettle, *C. chiopterus* (Meigen) and *C. dewulfi* Goetghebuer.

Prior to the bluetongue epidemic, research on *Culicoides* ecology in northern and central Europe was strongly limited, e.g., it was not clear where the immature stages of the Obsoletus Group in the affected regions develop. The massive losses among farmed ruminants caused by the bluetongue disease – Conraths et al. (2012, review) estimated a financial impact of 254 million Euros for Germany from 2006 to 2008 – motivated an increase of studies on *Culicoides*. In Germany, several investigations concerned the distribution, seasonal activity or host feeding habits of the members of the Obsoletus Group (e.g., Balczun et al., 2009; Bartsch et al., 2009; Kiel et al., 2009). Naturally, a critical issue was the clarification of their role in BTV transmission (e.g., Hoffmann et al., 2009, see section 1.3). In consequence of the bluetongue epidemic, the knowledge on the ecology of *Culicoides* considerably increased. However, information on the breeding ecology was still strongly limited at the end of the decade. In 2013, contemporaneously with the data collection for this thesis, first investigations provided initial information on livestock-associated breeding sites of *Culicoides* (e.g., González et al., 2013; Thompson et al., 2013; Zimmer et al., 2013a). Nevertheless, there are still wide knowledge gaps in this research field.

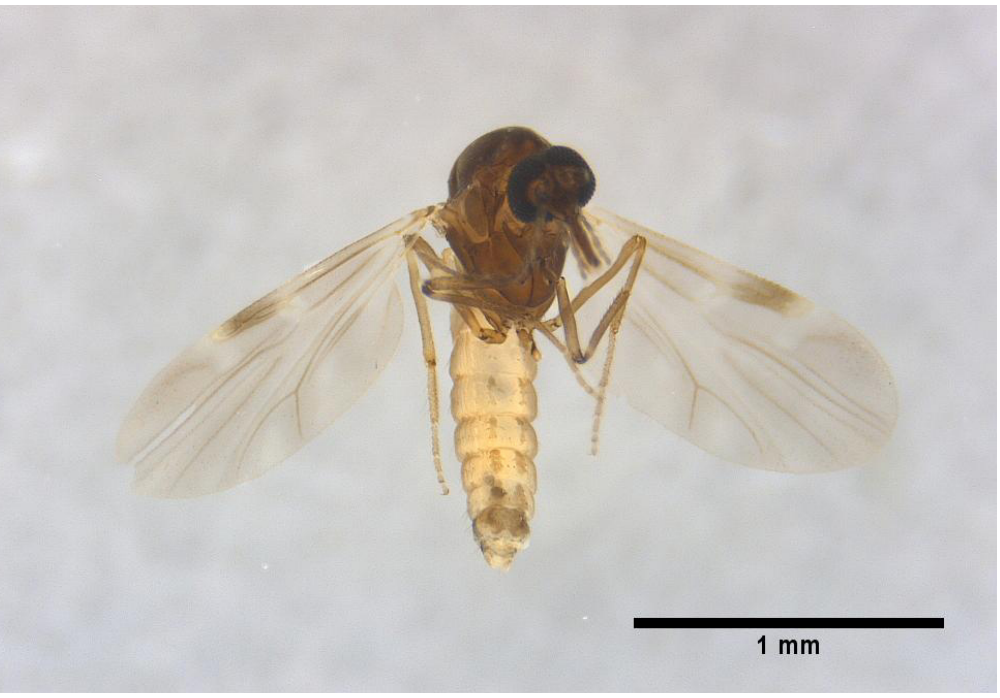

Figure 1.1 Female *Culicoides* (Obsoletus complex).

Before addressing the main questions of this thesis, section 1.1 will summarise the taxonomic terminology regarding the Obsoletus Group and clarify the definitions used throughout this thesis. Moreover, a general introduction to the bionomics of *Culicoides* (section 1.2) will prepare the widely ecological focus of the present work. The significance of *Culicoides* as pathogens vectors will subsequently be introduced with particular reference to the species of this group, against the background of their capability to transmit and spread diseases (section 1.3). Finally, the last section of the introduction will expound the need for a comprehensive research on breeding habitats and the development of *Culicoides* and summarise the questions aimed to be answered, in order to contribute to a deeper understanding of the ecology of these insects.

1.1 TAXONOMIC TERMINOLOGY OF THE OBSOLETUS GROUP

In northern Europe, the term "Obsoletus Group" widely refers to four morphologically close species within the subgenus *Avaritia* Fox, namely *C. obsoletus* s.s., *C. scoticus*, *C. chiopterus* and *C. dewulfi*. However, the progress in the development of molecular identification methods gave rise to discussions on phylogenetic patterns within the Obsoletus Group that additionally contributed to the inconsistent use of taxonomic definitions and terminology throughout the literature. Some authors refer to the four species as "Obsoletus complex" (e.g., Carpenter et al., 2006), whereas this term is more commonly used for two morphologically very close species within this group i.e., *C. obsoletus* s.s. and *C. scoticus* (e.g., Meiswinkel et al., 2007; González et al., 2013; Zimmer et al., 2014). Meiswinkel et al. (2004b) refrained from denominating the four species as a group and referred to *C. obsoletus* s.s. and *C. scoticus* as the "Obsoletus complex s.s." and to the other species as the "Chiopterus complex" and the "Dewulfi complex". Moreover, it was suggested to place *C. dewulfi* (formerly often referred to as *C. pseudochiopterus*) apart from the other three species (Gomulski et al., 2005; Nolan et al., 2007; Schwenkenbecher and Piertney, 2009). Nevertheless, within the scope of the present thesis, it will be referred to *C. obsoletus* s.s, *C. scoticus*, *C. chiopterus* and *C. dewulfi* as the Obsoletus Group, according to the classification by Campbell and Pelham-Clinton (1960), while the term "Obsoletus complex" will refer to *C. obsoletus* s.s. and *C. scoticus*.

1.2 GENERAL BIONOMICS OF *CULICOIDES*

As characteristic for all Diptera, the development of *Culicoides* is holometabolic and involves the egg, larvae, pupae and imago. In temperate species, completion of the life cycle requires about a month, however, the duration varies within the genus and is highly temperature-dependent (Gutsevich, 1973 cited by Szadziewski et al., 1997; Veronesi et al., 2009). Autogeny is common in some species, but usually, host feeding is necessary for reproduction (Linley, 1983). In multivoltine species, including the members of the Obsoletus Group, one blood meal matures one batch of eggs (Kettle, 1962; Meiswinkel et al., 2014).

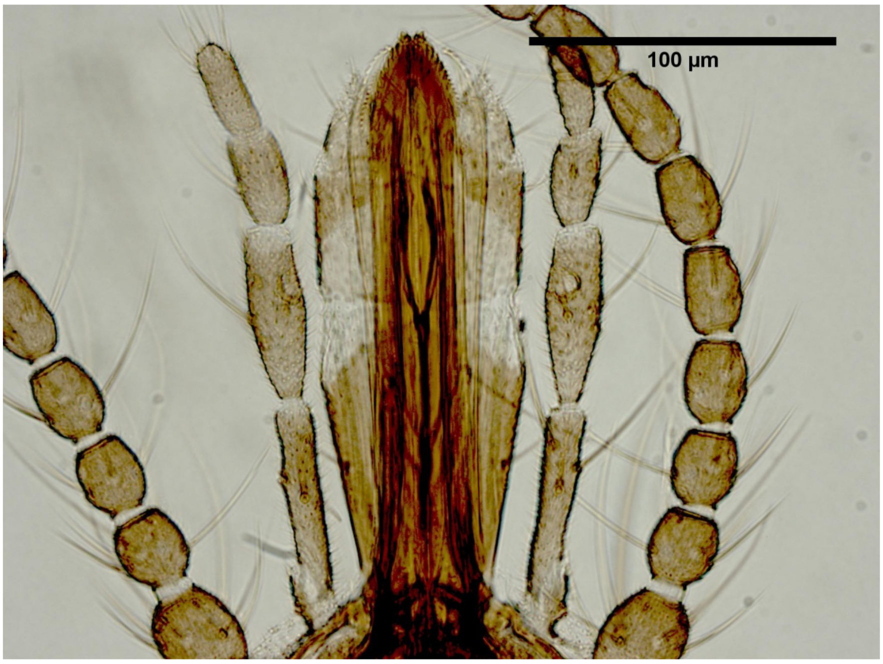

Figure 1.2 Mouthparts of a female *Culicoides* (*C. dewulfi*): blade-like proboscis and maxillae surrounded by the maxillary palps.

Many species are opportunistic feeders and will take their blood meal from different hosts, but upon availability, preferences are common, some species being classified as ornitophilic or mammalophilic (Kettle, 1962, 1977; Pettersson et al., 2013). All species of the Obsoletus Group feed on cattle, sheep, horses and goats. In addition, rabbits, deer or pigs occasionally serve as hosts and at least *C. obsoletus* s.s. feeds readily on birds and humans (Bennett, 1960; Jamnback, 1961; Nielsen, 1971; Carpenter et al., 2008a; Garros et al., 2011; Lassen et al., 2011; Ninio et al., 2011a; Martinez-de la Puente et al., 2012; Pettersson et al., 2013; Viennet et al., 2013). The processes involved in host attraction and location are not completely understood, but chemical odours and also visual cues are considered to play an important role (Blackwell, 2008; Logan et al., 2010). In *C. brevitarsis* Kieffer, initial attraction was suggested to depend on visual stimuli, while olfactory stimuli becomes important in short distances to confirm the nature of the suspected host (Bishop et al., 2008).

Mating, with or without mating swarms, can take place before or subsequent to the host-feeding. Oviposition usually takes place a few days later, the number of eggs varying strongly among species (Kettle, 1962, 1977; Yuval, 2006). Eggs are often laid in moist or wet substrates, as they do not withstand desiccation. The first of four larval instars hatches shortly after that, initiating the longest stage of the *Culicoides* life cycle (lasting between several days to weeks; Parker, 1950; Kettle, 1962; reviewed by Mellor et al., 2000). From the subsequent pupa, which is free but limited in mobility, the adult midge emerges after two to 10 days (Blackwell, 2008; Borkent, 2014).

Immature stages of most *Culicoides* develop in semiaquatic habitats. According to Kettle (1962, p. 404) the larvae are "neither genuinely aquatic nor terrestrial, but occupy the ecotone between [...]". However, various habitats are occupied by members of the genus, including water bodies, animal dung or decaying plant material (e.g., Kettle and Lawson, 1952; Mercer et al., 2003; Zimmer et al., 2014). The knowledge of food sources of *Culicoides* larvae is strongly limited but this life stage has been suggested to feed on algae, microorganisms or detritus (Kettle, 1962; Williams and Turner, 1976).

1.3 THE ROLE OF *CULICOIDES* AS PATHOGEN VECTORS

At least 50 viruses have been isolated from *Culicoides* midges worldwide (reviewed by Mellor et al., 2000). The limited number of pathogens transmitted to men includes the Oropouche virus in the Neotropical region (vector: *C. paraensis*; Tesh, 1994; Felippe-Bauer and Sternheim, 2008), however, the majority of viruses transmitted by *Culicoides* cause diseases in animals (reviewed by Mellor et al., 2000). Three are listed as important animal diseases by the World Organisation for Animal Health, i.e., the bluetongue disease, African horse sickness and epizootic hemorrhagic disease (OIE, 2016). The latter affects wild and domestic ruminants in parts of America, Asia and Australia. It causes a severe disease in deer but is often without severe clinical symptoms in other ruminant species (reviewed by Mellor et al., 2000). The main vectors are *C. variipennis* (Coquillet) in North America and *C. brevitarsis* in Australia (Foster et al., 1977; Parsonson and Snowdon, 1985). Transmitted by *C. imicola*, the African horse sickness virus causes a highly fatal disease in equines (reviewed by Mellor et al., 2000 and Coetzer and Guthrie, 2004).

Of major epidemiological relevance in Europe is BTV. While *C. imicola* is the main vector in South Europe and Africa, the members of the Pulicaris and Obsoletus Groups are responsible for virus transmission in northern and central Europe (reviewed by Wilson and Mellor, 2009). The bluetongue disease affects ruminants, mainly cattle and sheep, causing a variety of clinical signs including abortion, malformation of calves or lambs and milk drop (Osburn, 1994; Elbers et al., 2008). In 2007, 13.1% of affected cattle and 41.5% of affected sheep died in consequence of the disease in Germany (Conraths et al., 2009). Biting midges of the Obsoletus complex (*C. obsoletus* s.s. and *C. scoticus*) can be infected with BTV in the laboratory (Carpenter et al., 2006) and scientists isolated the virus from field-collected individuals in Germany (Mehlhorn et al., 2007). *Culicoides obsoletus* s.s. was strongly suggested to be a relevant vector after a comprehensive *Culicoides* monitoring in Germany, demonstrating high viral genome load in this species (Hoffmann et al., 2009). Moreover, entomologists found pools of *C. dewulfi* and *C. chiopterus* BTV-positive in the Netherlands (Meiswinkel et al., 2007; Dijkstra et al., 2008).

As sudden as the bluetongue epidemic, did a further viral disease emerge in 2011 in northern Europe (Rasmussen et al., 2012). Similar to BTV, the Schmallenberg virus causes malformations in calves and lambs. Further symptoms in affected animals are milk drop, diarrhoea or fever (Garigliany et al., 2012; Lievaart-Peterson et al., 2012). Again, species of the Obsoletus Group were suggested to be responsible for the transmission of the virus in the affected regions (De Regge et al., 2012; Elbers et al., 2013).

1.4 WHY STUDY *CULICOIDES* BREEDING ECOLOGY? – MOTIVATION AND AIMS OF THE THESIS

In current research, the majority of studies on *Culicoides* refer to adult populations and rely on data gained with blacklight traps, which is a standard tool for *Culicoides* surveillance (Meiswinkel et al., 2004b; Venter et al., 2009). The extensive use of this technique is reasonable, the operation being straightforward and requiring minimal effort of the researcher. Light traps allow easily standardised sampling in order to determine distributional ranges and abundance of species. Investigations of breeding habitats, e.g., with emergence traps, provide mostly punctual information in contrast to the comparably large attraction range of blacklight traps, although estimations of this range vary from two to 50 metres (e.g., Rigot and Gilbert, 2012; Kirkeby et al., 2013). Moreover, despite the convenience of this tool, there have been indications that the performance of light traps is not as accurate as widely supposed. Examples for criticisms are the limited use for estimations on vector-to-host ratios or that results are heavily influenced by the proximity to potential hosts (Lühken and Kiel, 2012; Elbers and Meiswinkel, 2014; Elbers and Meiswinkel, 2015). Furthermore, while in particular extraction techniques can be used to assess the immature density in a breeding substrate, light traps rather reflect *Culicoides* activity pattern.

Another major disadvantage of light trapping is that only a fraction of the adult *Culicoides* population is represented. While emergence traps sample males and females likewise, light traps primarily attract host-seeking females (Venter et al., 2009). In addition, abundances of certain species, e.g., of *C. chiopterus*, are underestimated (Carpenter et al., 2008a). Only recently, the comparison of light traps and sweep-netting revealed that the trapping success with these methods differs significantly among the species of the Obsoletus Group (Elbers and Meiswinkel, 2015).

Investigation of breeding sites can provide valuable information on abundances of immature stages of *Culicoides* and factors driving their development. This approach also provides manifold possibilities for research on ecology and behaviour. Such knowledge is the key to a successful control of adult populations. At present, most control measures target adult *Culicoides* and are often focused on the protection of livestock from being bitten by using insecticides on livestock, in stables or on insect screens (reviewed by Carpenter et al., 2008b and Mullens et al., 2015). However, the immature population offers alternatives for control measures. The presence of adult *Culicoides* – with the potential to transmit viruses – is primarily dependent on the availability of suitable breeding sites. Therefore, the first measure in order to reduce *Culicoides* populations ought to be the identification of important breeding habitats, in order to prevent their creation if possible, e.g., by an adapted farm management (Mellor and Wittmann, 2002; Carpenter et al., 2008b). Furthermore, existing breeding sites should be removed or modified to make them unsuitable for pre-adult development. The larval stage has the longest duration in the life cycle (Kettle, 1962) and is less mobile as compared to the adult midges. It therefore provides good opportunities for control measures. However, in order to suppress adult abundances successfully, we need to know as much as possible about the development of *Culicoides*. The knowledge of immature stages and their ecology is still alarmingly poor, as Mullens et al. (2015, review) recently pointed out. To contribute to a more comprehensive understanding of the breeding ecology of *Culicoides* is therefore the major aim of this work.

The present thesis focuses on several key aspects: particular emphasis is placed on methodical requirements of research on *Culicoides* breeding habitats, as comparable and effective study methods are the basis for meaningful research. Another aspect is the identification of important breeding sites and a better understanding of what characteristics make them suitable habitats for immature *Culicoides*. The impact of environmental parameters affecting the pre-adult development is a further principal component. The conducted studies focused on the species of the Obsoletus Group, being among the major vectors of BTV in northern and central Europe (see section 1.3). The main questions aimed to be answered with this thesis are summarised hereafter with references to the corresponding chapters and manuscripts.

Question 1 [chapter 3; manuscript I (→ 11.1.1)]: how comparable are methods available for the assessment of *Culicoides* abundances in breeding substrates?

Question 2 [chapter 3; manuscript II (→ 11.1.2)]: how does the trap design, i.e., colour and shape, impact the effectivity of emergence traps?

Question 3 [chapter 4; manuscript III (→ 11.1.3)]: what are the main breeding habitats of the species of the Obsoletus Group on cattle farms?

Question 4 [chapter 5; manuscript III (→ 11.1.3)]: what physico-chemical factors influence the presence and abundance of immature *Culicoides* in the main breeding habitats?

Question 5 [chapter 6; manuscript IV (→ 11.1.4)]: how does freezing of the breeding substrate impact the development success of overwintering *Culicoides* larvae?

Question 6 [chapter 7; manuscript V (→ 11.1.5)]: how does flooding impact the development success of larvae and pupae of *Culicoides*?

In chapter 8, the results of the presented studies will be referred to in a joint discussion with relation to these questions and the corresponding answers. The achieved results will be discussed in a more general and wider context, e.g., the choice of a suitable study method in the light of methodical requirements of *Culicoides* research, habitat binding and niche differentiation, or boundary points of the questions and results to climate change. Finally, it will be discussed **how the knowledge gained in the scope of this thesis can be applied for *Culicoides* control measures (Question 7).** Prior to presenting the studies, conducted to answer these questions, chapter 2 will cover the practical efforts, requirements and limits regarding the morphological identification of the species of the Obsoletus Group.

CHAPTER 2

MORPHOLOGICAL IDENTIFICATION OF THE OBSOLETUS GROUP SPECIES

The adequate *Culicoides* species identification belonged to the major requirements for the present thesis. Therefore, the investigation presented in this chapter, scrutinised morphological identification characters, suggested in the literature for differentiating the females of the Obsoletus Group. Based on the conclusions of this study, a short identification key for the females of this group will be presented in the last section of the chapter.

2.1 INTRODUCTION

The morphological identification of *Culicoides* is a challenging and often time-consuming task. In particular, the females of many species, including those of Obsoletus Group, are difficult to distinguish (Meiswinkel et al., 2004a; Nielsen and Kristensen, 2011). For the identification of the western Palaearctic *Culicoides* species, several dichotomous keys are available, i.e., Campbell and Pelham-Clinton (1960), Kremer (1965) and Delécolle (1985). While the males of the four species of the Obsoletus Group are identifiable by comparing characteristic features of the hypopygium, characters proposed by entomologists for discrimination of the females include the distinctness of the wing pattern, the shape of the maxillary palp or the spermatheca size. The keys distinguish between all species of the Obsoletus Group, however, the authors remark difficulties regarding the discrimination of *C. obsoletus* s.s. from *C. scoticus*. Delécolle (1985) differentiated the females of these species by differences of the spermathecae size and the orientation of the chitin plates surrounding the genital opening, but also showed that these diagnostic characters overlap. Campbell and Pelham-Clinton (1960) proposed other morphologic characters for the separation, e.g., the form of the third segment of the maxillary palp.

The emergence of BTV heavily necessitated methods for a reliable identification of the vector species. Therefore, an increasing number of studies emphasised the development of identification tools, often by seizing the characteristic features provided by Campbell and Pelham-Clinton (1960) or Delécolle (1985). The associated progress in the development of molecular identification techniques allowed for the evaluation of morphological features by comparing identification results with those of molecular approaches. In this context, the unreliability of the spermathecae size and genital structures, proposed by Delécolle (1985) to discriminate *C. obsoletus* s.s. and *C. scoticus* females, was affirmed by support of molecular identification methods (Pagès and i Monteys, 2005). A few years later, a further study described them as accurate for differentiation (Augot et al., 2010). In 2011, Nielsen and Kristensen published an identification key for the females of the Obsoletus Group, basically relying on the comparison of the shape of the third segment of the maxillary palp and the number of hairs on the first abdominal segment. Instead, a recent comprehensive online key on the females of the western Palaearctic *Culicoides* species does not separate between *C. obsoletus* s.s. and *C. scoticus* (Mathieu et al., 2012). Also newly developed techniques, as the computer-based analysis of wing geometrics, only allowed the separation of *C. dewulfi* and *C. chiopterus* (e.g., Henni et al., 2014).

The conflicting conclusions of entomologists regarding the suitability of identification characters required a close examination of their applicability. In order to allow for adequate species determination, female *Culicoides* of the Obsoletus Group were identified by molecular methods and – simultaneously – by morphological identification characters, proposed in the literature, in order to assess their practical applicability and reliability. This investigation included features of the *Culicoides* head (segments of maxillary palp, angle of the triangular field at upper eyes margin), wings (size, wing pattern) and the abdomen (spermathecae size, number of hairs on first abdominal tergite).

2.2 METHODS

The majority of the *Culicoides* females examined for this study were collected with emergence traps during the first sampling period of a field study, from different sample sites in Germany, which will be described in chapter 4 (Aug. - Sept. 2012, see section 4.2). Due to the absence of *C. scoticus* in these samples, a smaller number of individuals obtained with light traps were also included. The dissection of female *Culicoides* in 96% ethanol for slide-mounting followed the pre-identification as species of the Obsoletus Group (preserved in 70% ethanol for several weeks to months). Several body parts were placed into a drop of Euparal (3C 239, Waldeck, Münster, Germany) on an object slide: the wings, the head (dorso-ventral), the distal abdominal tip containing the spermathecae (ventro-dorsal) and the first abdominal tergite (dorso-ventral). A cover slip was carefully placed on the Euparal prior to drying the objects at 30 °C for at least 48 hours. The remaining parts of the body maintained in 96% ethanol for molecular identification.

The examination of some of the morphological characters in the slide-mounted specimens required using a microscope (Axiostar, Zeiss, Oberkochen, Germany) coupled with a microscope-camera (DFC 320, Leica, Solms, Germany in combination with the Leica Application Suite, version 4.5.0). Photographs were taken for morphometric measurements of several diagnostic features (Helicon Focus, version 5.2.7 and Image J, version 1.46r), e.g., the length and width of the wing. Table 2.1 provides the full list of examined parameters. Of the two wings, maxillary palps or spermathecae of an individual, the one in better condition after slide-mounting was used for these measurements. Selections of 170 female specimens were sent to the University of Bochum (Group Zoology /Parasitology) for molecular identification with a polymerase chain reaction (PCR)-based method (see Balczun et al., 2009 for methodical details). The results were utilised to assess the accuracy of morphological characters for the differentiation of the females of the Obsoletus Group. In order to verify the results of the molecular identification, this step was repeated for 98 individuals with a second PCR.

Table 2.1 Morphological identification features examined in the species of the Obsoletus Group, as proposed for species differentiation in the given references (CP = Campbell and Pelham-Clinton (1960); K = Kremer (1965); D = Delécolle (1985); A = Augot et al. (2010); N = Nielsen and Kristensen, 2011).

Identification criteria		**Specification**	**References**
Head	form of third segment of maxillary palp	e.g., triangular, bulgy, slender, cylindrical	CP, K, D, N
Head	ratio of third segment of maxillary palp	palp length/ palp width	N
Head	form of fourth segment of maxillary palp	e.g., short, stout	N
Head	ratio of fourth segment of maxillary palp	palp length/ palp width	
Head	angle of the triangular field at upper eyes margin	degree	CP
Wing	length	arculus to tip [mm]	CP
Wing	width	at widest point [mm]	
Wing	ratio	wing length/ wing width	
Wing	pattern	weak, medium, distinct	N
Abdomen	spermatheca size (interspecific differences)	at widest point [µm]	D, A
Abdomen	spermatheca size (intraspecific differences)	two spermathecae of equal or unequal size	CP, K
Abdomen	number of hairs on first abdominal tergite	per side	CP, N

2.3 RESULTS AND DISCUSSION

2.3.1 MOLECULAR SPECIES IDENTIFICATION

Of the 170 females, sent in for molecular identification, a total of 136 *Culicoides* females served for the analysis of morphological differentiation characters. The remaining 34 were excluded for the following reasons: in four cases, no DNA could be amplified and for 19 individuals molecular analysis produced ambiguous results. Further 11 individuals were identified as either *C. obsoletus* s.s. or *C. scoticus* in the first molecular identification attempt and as the other one of the two species in the second.

Nineteen females had been identified as *C. chiopterus* morphologically, but as *C. scoticus* by using molecular methods. Photographs of identification features were therefore sent to Dr. Bruno Mathieu, the primary author of the recently published online identification key on females of western Palaearctic *Culicoides* species (Mathieu et al., 2012). Dr. Mathieu confirmed the morphological identification as *C. chiopterus*, as did the second PCR. Therefore, these individuals remained in the group of analyses individuals, comprising 72 midges identified as *C. obsoletus* s.s, 24 as *C. scoticus*, 24 as *C. chiopterus* and 16 as *C. dewulfi*.

2.3.2 MORPHOLOGICAL IDENTIFICATION FEATURES OF THE HEAD

The maxillary palps (Figure 2.1) have been applied by several researchers for species differentiation. Delécolle (1985) describes the form of the third segment (MP3) as cylindrical and slightly bulged (*C. dewulfi*) or bulged (*C. obsoletus* s.s. and *C. scoticus*). According to Campbell and Pelham-Clinton (1960), the MP3 is elongate, slender and subcylindrical in *C. scoticus* and shorter and slightly inflated in *C. obsoletus* s.s.. Nielsen and Kristensen (2011) provide a more detailed description of this feature. They discriminated the females of the Obsoletus Group by comparing the ratio of length and width of this segment. The average values given by the authors are very close to those measured in the present study (Table 2.2), but the maximal and minimal limits distinguishing the species according to Nielsen and Kristensen (2011) could not be confirmed. In 18% of female *C. obsoletus* s.s. examined in this study, the ratio exceeded or equalled 2.6 and in 13% of *C. scoticus* the ratio was less or equal to 2.7. Moreover, Nielsen and Kristensen (2011) claimed that the differentiation can be done by visual inspections using the stereomicroscope without time-consuming slide-mounting. This seems implausible, considering that the proposed separation depends on a few microns only. Nevertheless, the shape of the MP3 remains a good species indicator.

Similar to the descriptions of Campbell and Pelham-Clinton (1960) or Nielsen and Kristensen (2011) this segment was short and triangular in *C. chiopterus* and much longer and cylindrical in *C. dewulfi* in the present study, allowing the identification of these two species. In *C. scoticus*, the segment often appeared slender and skewed while it looked usually more bulgy in *C. obsoletus* s.s.. However, there were several exceptions to this observation. Additionally to the MP3, the high ratio of the fourth segment of the maxillary palp (MP4) supported the differentiation of *C. dewulfi* from the three other species (Table 2.2). In addition, the MP4 was usually found to be noticeably short and stout in *C. chiopterus*, thus resembling the description by Nielsen and Kristensen (2011).

Remarks in the species description of Campbell and Pelham-Clinton (1960) and the key of Kremer (1965) refer to interspecific differences in the angle of the triangular field at the upper eyes margin. It was confirmed that the eyes meet at an obtuse angle in *C. chiopterus* and *C. dewulfi* and at a more acute angle in *C. obsoletus* s.s. and *C. scoticus* (Figure 2.2, Table 2.3). However, this character does not differ considerably in the latter two species.

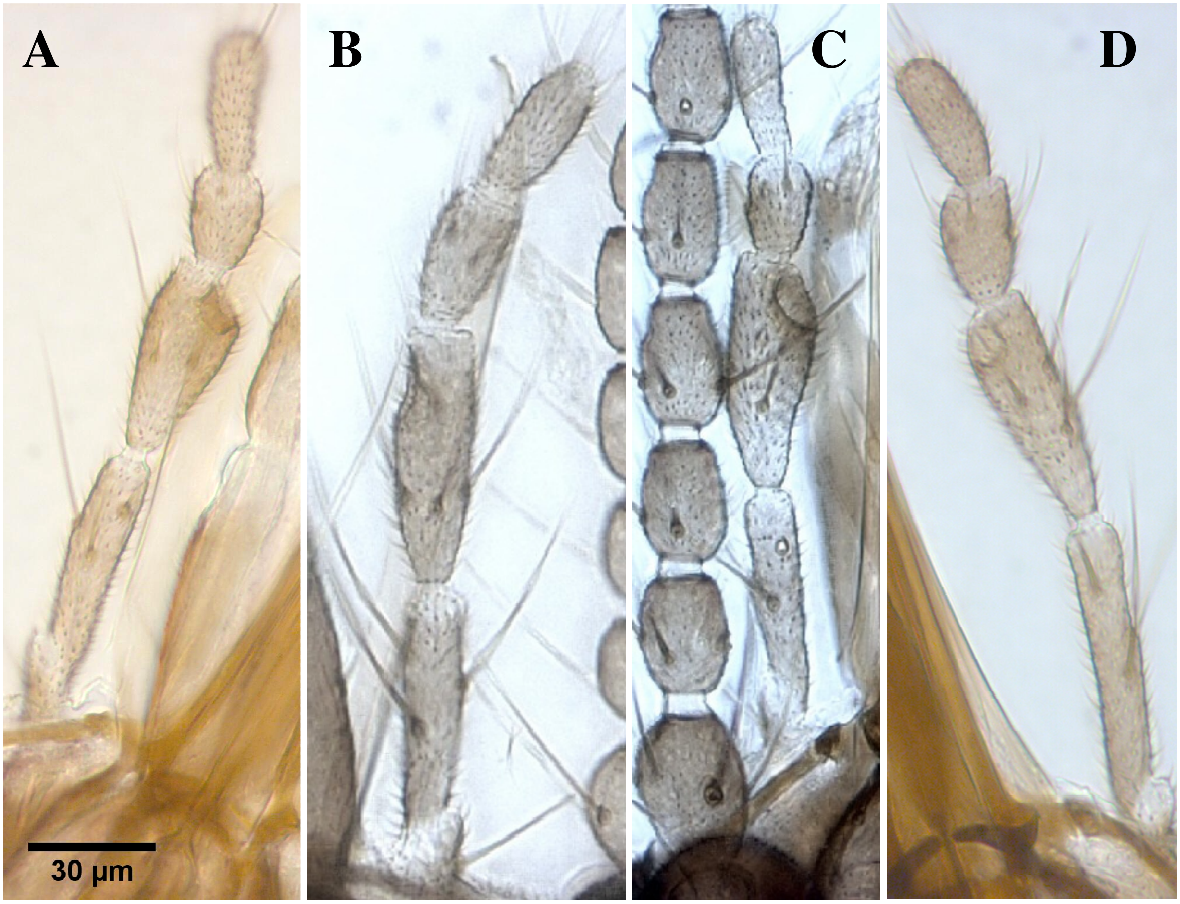

Figure 2.1 Maxillary palp of *C. chiopterus* (A), *C. dewulfi* (B), *C. obsoletus* s.s. (C) and *C. scoticus* (D).

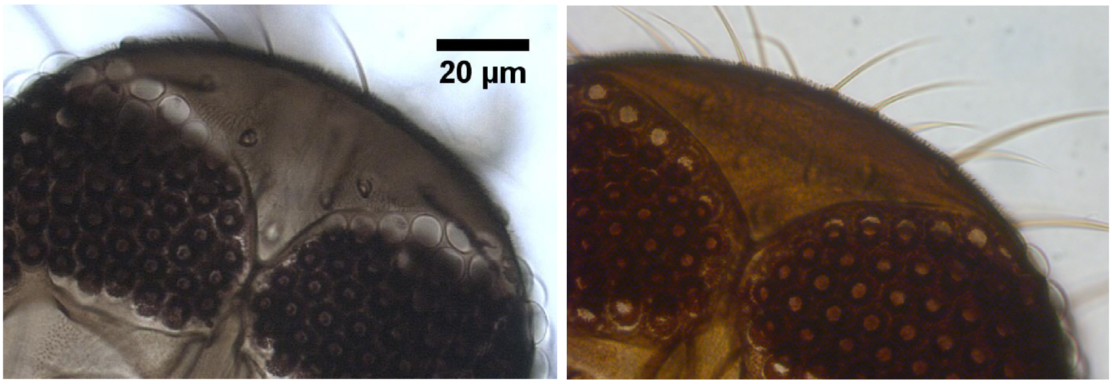

Figure 2.2 Angle of the triangular area at the upper eyes margin of *C. obsoletus* s.s. (A = acute angle) and *C. chiopterus* (B = obtuse angle).

Table 2.2 Minimal (min), mean (± standard deviation) and maximal (max) ratio of the length and width of the third segment (MP3) and the fourth segment (MP4) of the maxillary palp of the four species of the Obsoletus Group. The given values were measured in the present study (*) and by Nielsen and Kristensen (2011) (^{N}) (n = number of examined specimens).

Species	**MP3 Ratio**				**MP4 Ratio**			
	n	min	max	mean	n	min	max	mean
*C. chiopterus**	23	1.7	2.6	2.3 ± 0.2	23	1.0	1.8	1.4 ± 0.2
C. chiopterus^{N}	10		2.5	2.2 ± 0.2				
*C. dewulfi**	16	2.5	3.2	2.9 ± 0.2	16	1.6	2.7	2.0 ± 0.3
C. dewulfi^{N}	10	2.7		2.8 ± 0.2				
C. obsoletus s.s.*	64	2.0	2.9	2.4 ± 0.2	61	1.1	2.2	1.7 ± 0.2
C. obsoletus s.s.^{N}	20		2.6	2.4 ± 0.4				
*C. scoticus**	23	2.4	3.5	2.9 ± 0.2	21	0.8	2.2	1.8 ± 0.3
C. scoticus^{N}	20	2.7		2.9 ± 0.2				

Table 2.3 Minimal (min), mean (± standard deviation) and maximal (max) width of the angle of the triangular field at the upper eyes margin, measured in the four species of the Obsoletus Group (n = number of examined specimens).

Species	**Eyes angle (°)**			
	n	min	max	mean
C. chiopterus	21	78	100	90 ± 6
C. dewulfi	16	63	85	76 ± 6
C. obsoletus s.s.	63	40	82	62 ± 11
C. scoticus	18	44	94	69 ± 12

2.3.3 MORPHOLOGICAL IDENTIFICATION FEATURES OF THE WINGS

The wing length has been mentioned by several entomologists as a supportive character for the separation of *C. chiopterus* from the other three group members. According to Campbell and Pelham-Clinton (1960), the wing length is usually less than 1.1 mm in this species, while this value is exceeded in *C. dewulfi*, *C. obsoletus* s.s. and *C. scoticus*. Such a clear distinctness was not evident in the present results, as wing sizes mostly resembled in the four species (Table 2.4). These results may be an artefact, related to the fact that a fraction of *Culicoides* collected with emergence traps was presumably trapped and preserved when freshly emerged – without being fully sclerotised. Wings of midges trapped with this method sometimes appeared milky and bloated. Such specimens were not used for this study. However, inconspicuous divergences might have remained unnoticed, possibly leading to biased results. In the only species that data from light trap and emergence trap collections were available for – *C. obsoletus* s.s. – the wings were slightly larger in species sampled with light traps (i.e., 1.1 vs. 1.0 mm in wing length). Moreover, *C. scoticus* had the largest wings within the group and all of the examined individuals of this species were had been collected with light traps.

Of much greater value for species differentiation was the distinctness of the wing pattern (Figure 2.3). Nielsen and Kristensen (2011) describe *C. chiopterus* as a small species with faint wings. Also in the present study, wing pattern of *C. chiopterus* appeared consistently weaker, as compared to the other three species. *C. obsoletus* s.s. or *C. scoticus* were both characterised by a distinct pattern while the wing markings of *C. dewulfi* were medium distinct.

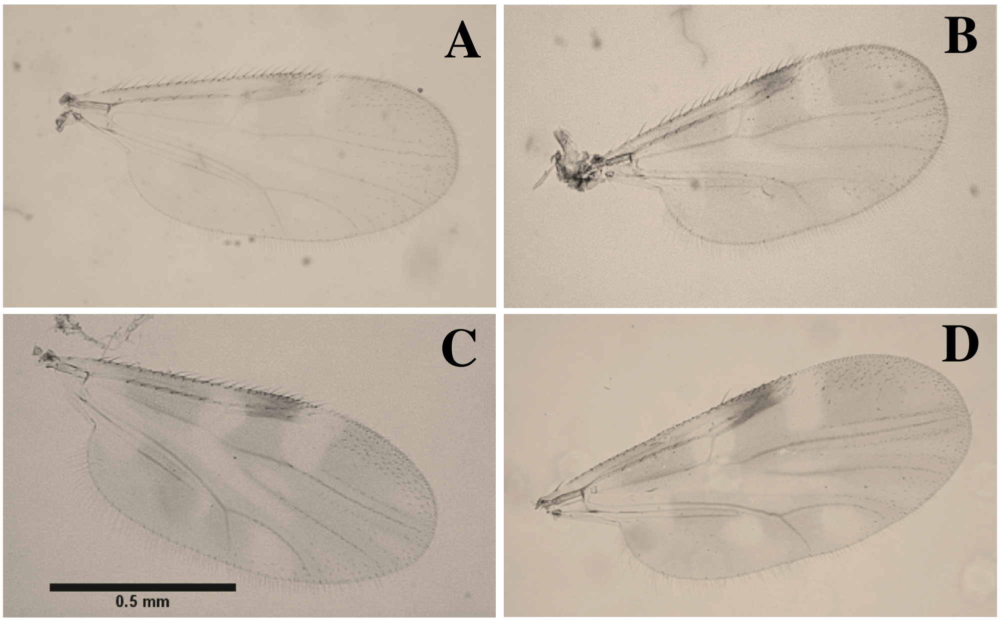

Figure 2.3 Wings of the Obsoletus Group species. The distinctness of wing patterns differs from weak (A = *C. chiopterus*) to medium (B = *C. dewulfi*) and distinct (C = *C. obsoletus* s.s.; D = *C. scoticus*).

Table 2.4 Minimal (min), mean (± standard deviation) and maximal (max) length and width of the wings measured in the four species of the Obsoletus Group. In addition, the wing ratio (length divided by width) and the distinctness of the wing pattern is given (n = number of examined specimens).

Species		**wing pattern**	**wing length [mm]**			**wing width [mm]**			**wing index**
	n		min	max	mean	min	max	mean	mean
C. chiopterus	15	weak	0.8	1.2	1.0 ± 0.1	0.4	0.6	0.5 ± 0.1	2.1 ± 0.1
C. dewulfi	11	medium	0.9	1.2	1.0 ± 0.1	0.4	0.6	0.5 ± 0.0	2.1 ± 0.1
C. obsoletus s.s.	52	distinct	0.8	1.2	1.0 ± 0.1	0.4	0.6	0.5 ± 0.0	2.1 ± 0.1
C. scoticus	21	distinct	1.1	1.3	1.2 ± 0.1	0.5	0.6	0.6 ± 0.0	2.1 ± 0.1

2.3.4 MORPHOLOGICAL IDENTIFICATION FEATURES OF THE ABDOMEN

The spermatheca size was mentioned by several entomologists as a potential feature for species differentiation within the Obsoletus Group (Campbell and Pelham-Clinton, 1960; Kremer, 1965; Augot et al., 2010). In the present study, sufficient measurements of the spermatheca lengths were not possible in the majority of specimens, due to the often crumpled appearance – possibly also a result of freshly emerged individuals being conserved in preservation liquid. Therefore, the results only refer to a small fraction of individuals and usually to only one of the spermathecae. This is problematic insofar as also intraspecific differences in the size of this character are evident according to several studies. Delécolle (1985) and Augot et al. (2010) only used the size of the bigger of the two spermathecae to distinguish the females of *C. obsoletus* s.s. and *C. scoticus*. In the present study, spermathecae were often – but not consistently – smaller in *C. obsoletus* s.s. than in *C. scoticus*. Very large spermathecae (here > 75 µm) occurred only in the latter species (Table 2.5). Thus, this character seems to be sufficient for merely a fraction of the population. A similar conclusion was drawn by Augot et al. (2010).

Campbell and Pelham-Clinton (1960) and Kremer (1965) mention differences in spermatheca size only with regard to *C. chiopterus* and *C. dewulfi*, while Delécolle (1985) used this character to differentiate *C. dewulfi* from the other three species. A distinct difference of the spermatheca size of *C. dewulfi* – being obviously larger than in the other species of the Obsoletus Group – could be confirmed in the present study and this feature was found reliable for the identification of *C. dewulfi*. Instead, the chitinous plates surrounding the genital opening, suggested for the separation of *C. obsoletus* s.s. and *C. scoticus* by Augot et al., (2010) and Delécolle (1985) were not used in this study. These structures were often indistinct, particularly in individuals collected with emergence traps.

Table 2.5 Minimal (min), mean (± standard deviation) and maximal (max) spermatheca length of the members of the Obsoletus Group (measured at the widest point) and number of hairs on the sides of the first abdominal tergite (n = number of examined specimens).

Species	**Spermathecae length (µm)**				**Number of abd. hairs**			
	n	min	max	mean	n	min	max	mean
C. chiopterus	4	60	74	68 ± 6	20	2	5	3 ± 1
C. dewulfi			n.a.		14	6	14	9 ± 3
C. obsoletus s.s.	20	50	75	64 ± 7	63	2	5	3 ± 1
C. scoticus	17	76	98	85 ± 7	24	3	5	4 ± 1

In addition to the spermathecae size, also the number of hairs on the sides of the first abdominal tergite allows separation of *C. dewulfi* from the other three species. The exact range of hairs in this species provided by other researchers varies from eight to 11 (Kremer, 1965) or eight to 12 (Campbell and Pelham-Clinton, 1960). According to the present results – referring to a larger number of individuals – this range was wider (6 - 14 hairs per side, Table

2.5). However, it was still sufficient for species discrimination as the lowest number of hairs in *C. dewulfi* was six while it did not exceed five in any other species.

Nielsen and Kristensen (2011) separated *C. dewulfi* and *C. scoticus* by the number of hairs higher or lower than eight (ignoring specimens with exactly eight hairs). According to Campbell and Pelham-Clinton (1960), four to eight hairs may occur in both species of the Obsoletus complex, while in the present study the number of hairs in the complex species ranged from two to five. Overall, this character seems to underlie strong variation.

2.4 CONCLUSIONS AND IDENTIFICATION KEY

Using morphological methods, reliable species identification was found unproblematic for *C. chiopterus* and *C. dewulfi*. The latter species could be easily identified by means of the typical cylindrical form of the MP3, the distinct unequal size of the two spermathecae and the number of hairs on the abdominal tergite. For the identification of *C. chiopterus*, the weak wing pattern and the short and triangular MP3 proved to be sufficient and reliable morphological characters. These features are particularly useful as they can be observed using the binocular without time-consuming slide-mounting.

The species of the Obsoletus complex could be differentiated from *C. dewulfi* and *C. chiopterus* by the distinct wing pattern, but the reliable distinction of the females of *C. obsoletus* s.s. and *C. scoticus* is difficult. The form of the MP3 appeared to be the most valuable character. It was usually slender and often skewed in *C. scoticus* and comparably shorter and more bulgy in *C. obsoletus* s.s.. However, there were exceptions to this observation. Consequently, females of *C. scoticus* and *C. obsoletus* s.s. were not identified to species level in the following studies presented in this thesis. In consideration of the uncertainties regarding the molecular species identification, described in the methods and the result section unnoticed misidentification cannot be fully excluded. It shall therefore be noted at this point, that neither results of morphological, nor of molecular identification methods should be blindly relied on. Most reliable results will be provided by the combination of both techniques.

The characters found most appropriate and practicable for morphological identification are hereafter summarised in a short identification key. Features requiring slide mounting for a sufficient evaluation are given in square brackets. If the spermathecae are not visible through the cuticula, slide-mounting or dissection of the abdomen is necessary. The comparison of the form of the MP3 in *C. chiopterus* and *C. dewulfi* without slide-mounting is possible, but requires some practice. The key also includes the females of *C. obsoletus* s.s. and *C. scoticus*, whereas it must be kept in mind, that the accurate separation cannot be assured of for 100% of cases and will moreover require some experience of the researcher.

Morphological identification key for the females of the Obsoletus Group

1A Wing pattern weak (compare Figure 2.3 A) OR wing pattern medium (compare Figure 2.3 B) and at least 6 hairs on each side of the first abdominal tergite, [upper eyes margins meeting at obtuse angle (compare Figure 2.2 B)]..2
1B Wing pattern distinct (compare Figure 2.3 C + D), [upper eyes margin meeting at acute angle (compare Figure 2.2 A)]...3

2A Wing pattern medium (compare Figure 2.3 B), at least 6 hairs on each side of the first abdominal tergite, the two spermathecae of distinct unequal size, MP3 of elongated and cylindrical appearance (compare Figure 2.1 B)..***C. dewulfi***
2B Wing pattern weak (compare Figure 2.3 A), MP3 short and bulgy, sometimes of triangular appearance (compare Figure 2.1 A)...***C. chiopterus***

3A [MP3 bulgy (compare Figure 2.1 C)]...***C. obsoletus* s.s.**

3B [MP3 slender, sometimes of skewed appearance (compare Figure 2.1 D)]..........***C. scoticus***

CHAPTER 3

STUDYING *CULICOIDES* BREEDING ECOLOGY: METHODICAL ASPECTS

Comparable and adequate research methods are an essential requisite for any successful research. Therefore, chapter 3 is dedicated to the evaluation of sampling methods available for the investigation of *Culicoides* breeding habitats. Two studies will be presented, followed by a joint discussion of the results: a comparison of emergence traps and immature extraction techniques was conducted while a second investigation emphasised the evaluation of different designs of emergence traps, in order to estimate the impact of colour and shape on the trapping efficiency.

This chapter is based on two manuscripts, of which one has been published by the journal **Veterinary Parasitology** [→ manuscript I (*Assessment of the abundance of Culicoides chiopterus and Culicoides dewulfi in bovine dung: a comparison of larvae extraction techniques and emergence traps*), section 11.1.1)]. The second work is, at the time of thesis submission, under review for publication in **Veterinary Parasitology** [→ manuscript II (*Comparison of emergence traps of different colour and shape in trapping of Culicoides (Diptera: Ceratopogonidae*), section 11.1.2].

3.1 INTRODUCTION

Kettle and Lawson (1952), who studied immature stages of British *Culicoides*, classified five types of breeding sites: bogland (i), fresh water marsh (ii), swamp (iii), mud (iv), salt marsh (v) and dung (vi). However, the range of breeding sites, described to date, exceed these categories by far. Very diverse substrates are occupied by the species of this genus, including intertidal sand, fungi, dairy wastewater ponds, tree holes and various kinds of decaying plant material, e.g., cornstalks, banana stem cells or leaf litter (Jamnback, 1965; Linley and Mook, 1975; Hackman and Meinander, 1979; Mullens and Rodriguez, 1989; Pappas and Pappas, 1990; Mercer et al., 2003; González et al., 2013). In order to gain information on immature stages in potential breeding substrates, two basic strategies can be applied: one can either extract the larvae or pupae or wait for the emergence of adult midges. Knowledge on how the efficiency of extraction techniques compares to that of emergence traps, is limited. Therefore, the first experiment presented in this chapter, aimed to compare two extraction methods to that of emergence traps, in order to determine the abundance of *C. chiopterus* and *C. dewulfi* in cowpats.

Several techniques are available for the extraction of juveniles, such as sieving, decanting, Berlese funnel-extraction, agar-extraction and flotation (reviewed by Hribar, 1990). For the application of flotation techniques, breeding substrates are mixed with solutions with a high specific gravity, resulting in the flotation of organic material to the solution surface – including immature stages – while inorganic material settles at the bottom. Different kinds of suspension have been successfully used for sampling of *Culicoides*, e.g., with solutions of sugar, magnesium sulphate or sodium chloride (Ladell, 1936; Wirth, 1952; Kettle and Lawson, 1952; Dyce and Murray, 1966; Blanton and Wirth, 1979; Mullens and Rodriguez, 1984; Blackwell and King, 1997; Zimmer et al., 2013a). Sugar-flotation is considered effective and less harmful to immature stages as compared to other flotation techniques, e.g., those utilising salt-solutions (reviewed by Hribar, 1990), and was therefore included in this study. Larvae of the Obsoletus complex have been previously extracted via sugar-flotation from dungheap material (see Manuscript VI, section 11.2.1), but to date, no study used the technique on cowpats.

The second extraction method tested in the present study was a modified version of the Berlese- or Tullgren-funnels. Several authors successfully applied this technique to recover immature *Culicoides* (Jamnback and Wirth, 1963; Jamnback, 1965; Kline et al., 1975; Blanton and Wirth, 1979; Blackwell et al., 1999). According to Hribar (1990, review), Berlese is a useful method to extract larvae from different substrates, e.g., aquatic plants, rotting leaves or mosses. However, it is unknown if it sufficiently extracts *Culicoides* from animal dung.

Instead of extracting immature stages from breeding habitats, adult *Culicoides* midges can be collected from substrates when emerging. Emergence traps are commonly used in current research (e.g., Kirkeby et al., 2009; Foxi and Delrio, 2010; González et al., 2013; Harrup et al., 2013; Thompson et al., 2013), also in order to sample species of the Obsoletus Group from cow dung, i.e., from dungheap material as well as from cowpats in previous investigations (Thompson et al., 2013; see Manuscript VI, section 11.2.1). A direct comparison of these three methods should allow a better interpretation of the results gained with the different techniques and thus, provide essential knowledge facilitating the choice of methods in future studies.

Aside from the lack of knowledge on the comparability to other methods, the significance of emergence traps in research on Diptera is beyond doubt. Therefore, a second experimental study was conducted in order to identify factors with impact on the effectivity of this tool, and thus, provide information necessary to optimise future trap constructions. A variety of different trap designs have been applied in previous studies to sample emerging *Culicoides*, e.g., constructions of quadratic wooden boxes or tarred roof paper cones (Dove et al., 1932; Davies, 1966; Braverman, 1970). Further designs are tent-like traps of white netting, plastic buckets, black cardboard cones or simple ice-cream container (Battle and Turner, 1972; Pajor, 1987; Dyce and Marshall, 1989; Bishop et al., 1996a, 1996b, 2005; Uslu and Dik, 2010).

Despite the variety of trap types used in the past, nothing is known about the relevance of the trap shape for the trapping success. Therefore, in order to estimate the potential impact of this factor, a quadratic trap (also used in the first experiment of this chapter) was directly compared to a cone-shaped trap. Moreover, the experiment incorporated traps of two different colours. To facilitate the sampling of *Culicoides*, emergence traps are often used in combination with transparent collection containers, utilising the positive phototaxis of adult midges (Megahed, 1956; Becker, 1960; Bidlingmayer, 1961). A dark and non-transparent trap body, maximising the differences in light intensity between trap and collection container, was therefore expected to increase the trapping success. Thus, the number of *Culicoides*, emerging from cowpats and trapped in black-coloured traps of both shapes – conical and quadratic – was compared to the number of trapped individuals in white-coloured traps.

3.2 METHODS

3.2.1 SAMPLE SITE

The cowpat samples for all experimental studies in the scope of this thesis were collected at an organic cattle farm in north-western Germany (GPS coordinates: N53 9.985, E8 8.771). The farm holds an approximate number of 110 dairy cows plus a fluctuating number of heifers. The pasture, that provided the cowpats, is surrounded by trees and a motor highway and is located at a distance of 160 metres from the main cowshed of the farm. The sample site was only a few kilometres away from the laboratory, allowing the transport of samples within approximately 10 minutes.

3.2.2 EMERGENCE TRAPS

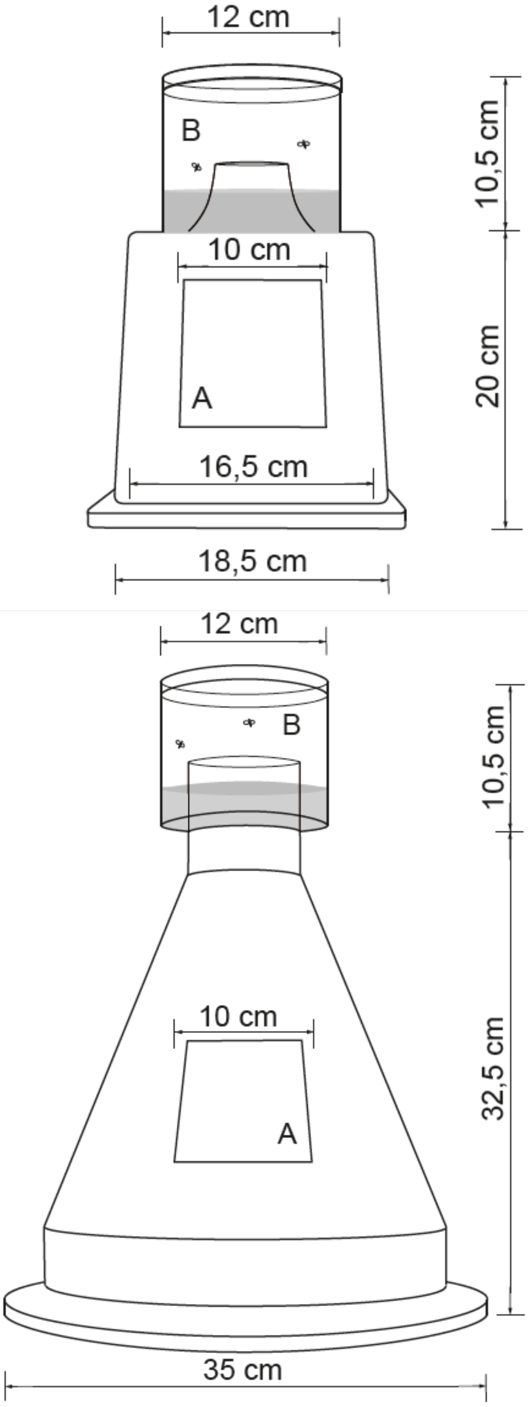

Figure 3.1 Schematic drawing of the quadratic (top) and cone-shaped emergence traps (bottom). Aeration windows (A) are covered with gauze (mesh size: 105 µm). Emerging insects are trapped and preserved in the salt solution in the transparent collection container (B).

The quadratic emergence traps (Figure 3.1) used in both experiments composed of a reversed plastic bucket (5.9 L) of white colour. Two lateral aeration windows (10x10 cm), covered with gauze (mesh size: 105 µm), allowed for air ventilation. The bucket was connected through a hole with a transparent top collection container (material: polystyrene, LICEFA, Germany, Bad Salzuflen). Cone-shaped traps (Figure 3.1), constructed from reversed white plastic funnels were combined with the equivalent collection container and aeration windows. Approximately 50 ml 20%

NaCl saline, supplemented with a drop of detergent, preserved emerging insects flying into the top container. After moistening the (cowpat-) samples within the traps every second to third day with tap water (approximately 20 ml per sample), using a spray diffuser, all emerged insects were collected from the saline, using forceps or a pipette. Emerged *Culicoides* were sorted out and preserved in ethanol (70%) for further identification. This procedure usually continued until no *Culicoides* emerged for at least 14 consecutive days.

3.2.3 BERLESE

The Berlese-funnel, also known as Tullgren-funnel, is a common technique for the extraction of organisms from soil samples: a funnel contains a soil sample and a temperature gradient is created with a heat source. The original method described by Antonio Berlese (1905) utilises a water jacket as a heat source while Albert Tullgren (1908) used an electric light bulb instead (cited by Vogt et al., 2015). In the present design, dung samples were evenly spread on coarse-meshed gauze fixed between two plastic cylinders (used instead of funnels to avoid the danger of larvae sticking to the funnel neck). These were set upon a petri dish filled with tap water, with a halogen lamp (57 W) fastened above at a wooden frame, designed to process a maximum of twelve samples at the same time. Immature stages within the substrate that migrated downwards fell into the water in the petri dish. This modified Berlese device (Figure 3.2), used to extract immature *Culicoides* from cowpats, will be referred to as "Berlese" throughout this thesis.

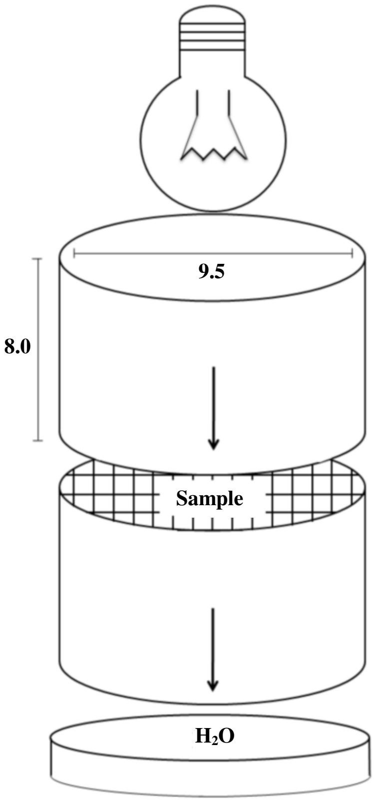

Figure 3.2 Schematic drawing of the applied Berlese device. The sample is spread on coarse meshed gauze (ø 0.5 cm) fixed between two plastic cylinders, set upon a petri dish filled with tap water (distance sample to water surface ~6 cm). A halogen-lamp (57 W) is placed above (distance to sample ~10.7 cm).

3.2.4 COMPARISON OF METHODS: BERLESE, SUGAR-FLOTATION AND EMERGENCE TRAPS

3.2.4.1 SAMPLING AND EXPERIMENTAL SETUP

The comparison of flotation, Berlese and emergence traps implied the mixing of cowpats in order to maximise the uniformity of subsamples compared. In order to exclude that this alteration of the breeding substrate affects the number of individuals emerging, a preliminary study was conducted, comparing the emergence of *Culicoides* from mixed and unaltered cowpat subsamples. The results illustrated that the mixing does not affect the development success of *C. chiopterus* and *C. dewulfi*. For details of this experiment see manuscript I (section 11.1.1).

In January 2014, 10 cowpats were collected, in order to compare the methods. The sampling aimed at the overwintering population. Since many *Culicoides* species in temperate regions overwinter as larvae (Becker, 1960; Jones, 1967; Szadziewski et al., 1997), the immature *Culicoides* in cowpats were expected to be in this stage. Last grazing by cattle had taken place about four months earlier, which was therefore expected the minimal age of collected samples. After the transport to the laboratory, each pat was thoroughly mixed and 100 g of every pat were placed in the Berlese device. Miniature data loggers (ø = 1cm; iButton DS1921G, Maxim Integrated, San Jose, CA, USA) monitored the temperatures within these samples during the extraction process. The daily collection of *Culicoides* larvae (every 24 hours) continued until no additional larvae appeared for two consecutive days.

Replacing the water within the petri dishes ought to prevent toxication through dying organisms extracted from the cowpats. Five of the ten cowpats, from that juvenile *Culicoides* were extracted, served for the comparison of the three methods, i.e., Berlese, emergence traps and sugar-flotation. The latter method was tested with 200 g from these five pats: after rinsing subsamples of 20 g over a sieve cascade (sieves: 2 mm and 63 µm mesh size), the content of the last sieve (63 µm) was mixed in a dish (24.5 cm × 10 cm) with 100 ml saturated sugar solution. In a screening of these flotation mixtures with a stereo microscope (10x), *Culicoides* larvae were collected with a pipette, counted and preserved in ethanol (70%). In order to sample emerging *Culicoides*, another 200 g of each of the five cowpats was placed under the quadratic emergence traps, described in section 3.2.2. These samples maintained at a rearing temperature of 20.3 ± 1.4 °C (mean ± standard deviation) under natural daylight conditions.

Two weeks after this first experimental unit, the entire procedure was repeated, including the collection of cowpats, testing via Berlese, the selection of five "positive" samples (i.e., cowpats containing *Culicoides*) and the examination of 200 g cowpat subsamples with each of the three methods. After 73 days (first experimental unit) and 59 days (second experimental unit), no more *Culicoides* had been found in the emergence traps for two consecutive weeks and this experiment was stopped.

3.2.4.2 IDENTIFICATION AND REARING OF *CULICOIDES*

The *Culicoides* larvae extracted with sugar-flotation and Berlese were identified to species level according to the colour and shape of head capsules given in the key of Kettle and Lawson (1952). Moreover, the rearing of 257 individuals collected via Berlese (180 *C. chiopterus* larvae and 77 *C. dewulfi* larvae) aimed to verify the morphological identification of larvae. The substrate for this purpose consisted of dried and pestled cowpat material, previously frozen at -30 °C degree for 48 hours. This remoistened substrate (ratio dry material: tap water = 1:3) and filled into small petri dishes (60x15 mm, filled to one-third). Groups of ten larvae, morphologically identified as either *C. chiopterus* or *C. dewulfi*, were transferred to the dishes and maintained at room temperature (20.3 ± 1.4 °C) and natural daylight conditions. For the removal of eclosed imagos, the dishes were placed into a small gauze bag and opened until all midges had left the dish, attracted by the light of a near window. This daily procedure was continued until all larvae within a dish had emerged successfully or until no emergence had occurred for at least 14 subsequent days. Adult *Culicoides* females were identified following the key introduced in chapter 2 (see section 2.4). Males were identified according to the key of Campbell and Pelham-Clinton (1960).

3.2.4.3 STATISTICAL ANALYSIS

All statistical analyses were carried out with the program R (R Core Team, 2011), using confidence levels of 5% to define statistically significant differences. A one-way repeated measures ANOVA, with the fixed factor "method" and the random factor "pat", compared the individual numbers obtained with the three methods for *C. chiopterus* and *C. dewulfi* separately. In addition, paired t-tests compared the method results and Spearman’s rho rank correlation coefficient (ρ) was calculated using the function *corr.test* from the R-package "psych" (version 1.0 - 97, Revelle, 2011) for all possible pairwise comparisons (emergence/flotation, emergence/Berlese, flotation/Berlese). *P*-values obtained with t-tests and correlation coefficients were corrected after Bonferroni to take multiple comparisons into account.

3.2.5 COMPARISON OF EMERGENCE TRAPS OF DIFFERENT COLOUR AND SHAPE IN TRAPPING OF *CULICOIDES*

3.2.5.1 SAMPLING AND EXPERIMENTAL SETUP

The sampling of cowpats for this experiment took place in June 2014. Two days in advance of the main experiment, small subsamples (approximately 10 g each) were collected from the edges of 25 cowpats for the extraction of *Culicoides* larvae with the Berlese. This measure ensured the inclusion of cowpats containing immature stages in the final experiment. Of ten "positive" cowpats, a 14x14 cm square was cut out and divided into four subsamples (7x7 cm). The four subsamples of each pat were placed in four different emergence trap types: cone-shaped white-coloured (i), cone-shaped black-coloured (ii), quadratic white-coloured (iii) and quadratic black-coloured (iv). Ten traps of each shape (conical and quadratic) were masked with a non-transparent black foil for the black-coloured trap versions.

Data loggers (HOBO Pro v2, Onset, Bourne, MA, USA), fastened inside 12 emergence traps (three loggers per trap type) measured air temperature and humidity hourly. Additionally, miniature temperature loggers (iButton DS1921G, Maxim Integrated, Sunnyvale, CA, USA), carefully inserted into the centre of the 12 cowpat subsamples, monitored the substrate temperature. The traps were placed outdoors on a mowed lawn and secured with tent pegs for stabilisation. The light intensity at the bottom of all traps was measured once during the experiment (between 12 and 3 pm) with a lux meter (PeakTech® 5025, PeakTech, Ahrensburg, Germany). A weather station at a distance of 1.2 kilometres from the test site (University of Oldenburg, www.uni-oldenburg.de/wetter) provided information on the ambient temperature. *Culicoides* midges were sorted out after emergence and identified to species level following the key introduced in chapter 2 (see section 2.4, females) and the key of Campbell and Pelham-Clinton (1960, males). The experiment ended after 46 days, subsequent to two weeks without emergence of any *Culicoides*.

3.2.5.2 STATISTICAL ANALYSIS

Potential relations between the number of trapped individuals (response variables: "all *Culicoides*", "*C. chiopterus*" or "*C. dewulfi*") and the colour and shape of the emergence traps as explanatory variables were investigated by applying linear mixed-effects models (function *lme*, package "nlme", version 3.1 - 120, Pinheiro et al., 2015; R Core Team, 2014). The ID of each cowpat was included as the random effect "pat" in the model to take variations of the *Culicoides* abundance in different cowpats into account. After the exclusion of a fixed effect, i.e., "colour" or "shape", an ANOVA compared the original and the new model. The models were run with and without outliers to check if their exclusion leads to distinct changes in *p*-values or coefficient estimates. A conditional coefficient of determination (R^2) and residual plots (histograms of Pearson residuals, fitted values vs. Pearson residuals) evaluated the final models (Nakagawa and Schielzeth, 2013; Johnson, 2014). A confidence level of 5% was used to define statistically significant differences.

3.3 RESULTS

3.3.1 COMPARISON OF METHODS: BERLESE, SUGAR-FLOTATION AND EMERGENCE TRAPS

All three methods successfully sampled larvae or adult *Culicoides* midges from all cowpats. Most individuals, i.e., 1,898 larvae, were obtained with the sugar-flotation method, while the Berlese method yielded 1,585 larvae (Table 3.1, Figure 3.3). With emergence traps, 1,117 adult *Culicoides* were collected. All individuals beside one female of the Obsoletus complex were identified as *C. chiopterus* (flotation: 59.7%, Berlese: 64.2%, emergence traps: 66.0%) or *C. dewulfi* (flotation: 39.8%, Berlese: 35.8%, emergence traps: 33.7%).

The one-way repeated measures ANOVA revealed a significant effect of the applied method for the number of individuals of *C. chiopterus* (F (2.9) = 6.7, $p < 0.01$) and *C. dewulfi* (F (2.9) = 5.2, $p < 0.05$). The t-tests partly supported this result with significant differences in individual numbers of *C. chiopterus* obtained with emergence traps and both extraction techniques. Spearman’s rho rank correlation coefficient (ρ) and the results of the paired t-tests are given in Table 3.2. Calculation of Spearman’s rho revealed that individual numbers obtained from the subsamples of the same cowpats using the three different methods strongly correlated positively in both species.

Examining a cowpat sample of 20 g using sugar-flotation, required averagely 22 ± 5 minutes. From samples in the Berlese device, larvae were detected at four days from the start of the experiment, but the majority of individuals were extracted during the first two days, i.e., 50.3% within the first day and 45.1% within the second day. The temperature in the Berlese samples increased from 23.6 ± 0.7 °C during the first 24 hours to 26.0 ± 3.5 °C on the fourth day.

The species identification of extracted larvae (Figure 3.4) and reared adults conformed in all cases and the head capsule measurements of the two species closely resembled those of Kettle and Lawson (1952, Table 3.3), referring to fourth instar larvae. Of the *C. chiopterus* larvae, 71% (of 180 larvae), and of the *C. dewulfi* larvae, 99% (of 77 larvae) were reared successfully.

Table 3.1 Total number and mean number (± standard deviation) of larvae extracted from cowpat subsamples with sugar-flotation and Berlese and total number and mean number of adults collected with emergence traps (n.d. = not determinable).

Method		***C. chiopterus***	***C. dewulfi***	**n.d.**
Sugar-flotation	total	1133	765	0
	mean	113.3 ± 65.9	75. 6 ± 84.2	
Berlese	total	1011	564	10
	mean	101.1 ± 50.3	56.4 ± 57.2	
Emergence trap	total	737	376	4
	mean	73.7 ± 53.0	37. 6 ± 44.1	

Table 3.2 Results of Spearman's rho rank correlation coefficient (ρ) and paired t-tests (df = 9 for all tests) to compare the individual numbers obtained from the subsamples of ten cowpats using sugar-flotation, Berlese and emergence traps.

Methods	**Spearman's rho**		**Student's t-test**	
	C. chiopterus	*C. dewulfi*	*C. chiopterus*	*C. dewulfi*
Emergence: flotation	$\rho = 0.85$, $p < 0.01$	$\rho = 0.73$, $p < 0.05$	t = 4.5, p < 0.01	t = 2.5, p = 0.10
Emergence: Berlese	$\rho = 0.84$, $p < 0.01$	$\rho = 0.75$, $p < 0.05$	t = 3.0, p < 0.05	t = 1.9, p = 0.26
Berlese: flotation	$\rho = 0.92$, $p < 0.001$	$\rho = 0.93$, $p < 0.001$	t = 0.9, p = 0.42	t = 2.0, p = 0.21

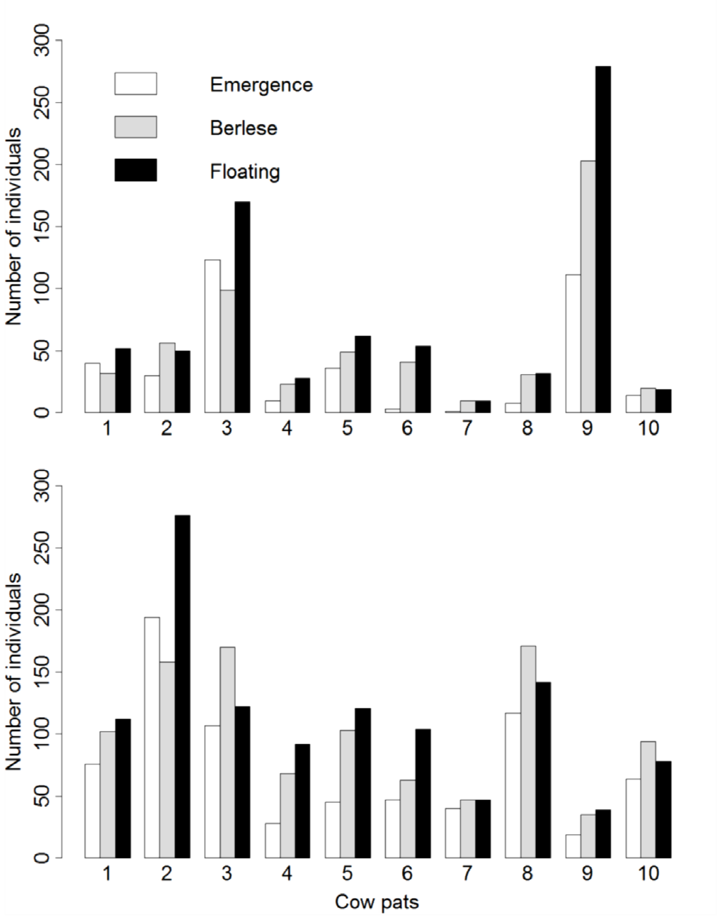

Figure 3.3 Number of larvae and adult individuals of *C. chiopterus* (bottom) and *C. dewulfi* (top) obtained via sugar-flotation, Berlese and emergence traps from the subsamples of ten cowpats.

Table 3.3 Length and width of head capsules (mean ± standard deviation) and head ratio (length/width) of the larvae of *C. chiopterus* and *C. dewulfi* measured in the present study (*) in comparison to the results of Kettle and Lawson (1952^{K}), measured in fourth instar larvae (n = number of examined specimens).

Species	**n**	**head length [µm]**	**head width [µm]**	**head ratio**
*C. dewulfi**	8	162 ± 6	123 ± 4	1.32 ± 0.04
C. dewulfi^{K}	8	173 ± 7	122 ± 3	1.33 ± 0.03
*C. chiopterus**	6	120 ± 2	103 ± 2	1.16 ± 0.02
C. chiopterus^{K}	24	115 ± 5	99 ± 4	1.16 ± 0.05

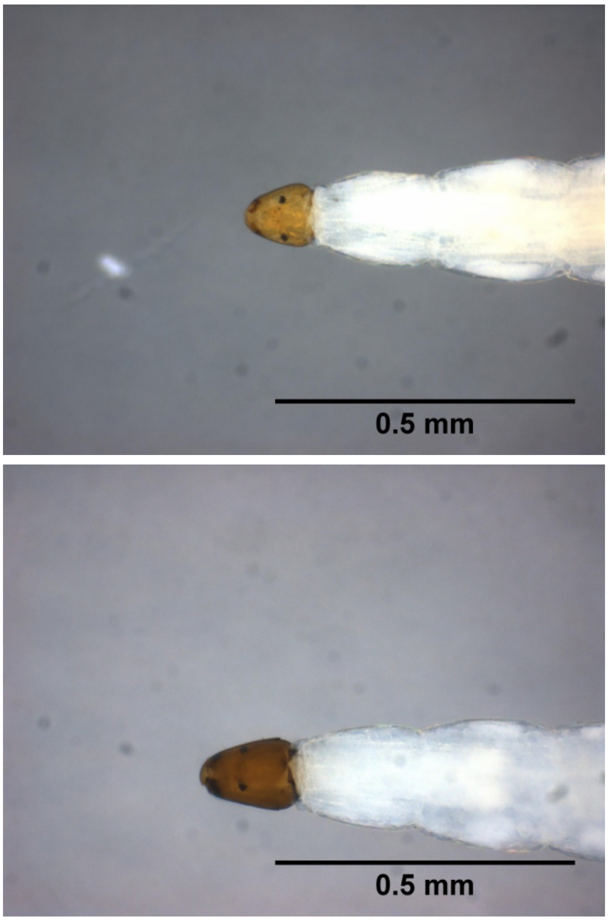

Figure 3.4 Head capsules of fourth instar larvae of *C. chiopterus* (A) and *C. dewulfi* (B).

3.3.2 COMPARISON OF EMERGENCE TRAPS OF DIFFERENT COLOUR AND SHAPE IN TRAPPING OF *CULICOIDES*

The 665 *Culicoides*, collected with emergence traps, were identified as *C. chiopterus* (464 specimens: 179 males, 285 females) and *C. dewulfi* (201 specimens: 33 males, 168 females). The total numbers of trapped *Culicoides* varied greatly among cowpats from six individuals from all subsamples of one pat to 179 midges. The quadratic white-coloured traps collected 70 *Culicoides* while 101 individuals were found in cone-shaped white-coloured traps. Overall, more individuals were trapped with black traps, i.e., 318 with the quadratic trap and 176 with the cone-shaped trap. According to the regression analysis, the trap colour explained a significant amount of variation in the number of trapped *Culicoides*. Instead, "shape" was dropped as insignificant from the model (Table 3.4). Black-coloured traps sampled higher numbers of individuals (Figure 3.5). For *C. chiopterus*, dropping of the fixed effect "shape" led to bad model fit (patterns in residual plots) and it was therefore retained in the model. For *C. dewulfi*, analysis of the full data set indicated a significant effect of "colour", but no effect of shape. However, the exclusion of two outliers in the response resulted in this variable being non-significant.

Temperature and relative humidity resembled within different trap types (Table 3.5). The ambient temperature and relative air humidity were on average 4.5 ± 7.0 °C and 10.8 ± 1.7%, respectively, lower than the average values within traps. The light intensity was more than three times higher in white trap bodies compared to black traps (Table 2).

Table 3.4 Regression coefficient estimates (± standard errors), standard deviation (SD) of the random effect "pat" and *p*-values for fixed effects retained in the linear mixed-effects models (*lme*), analysing the relation between trapped *Culicoides* and the colour and shape of emergence traps. Akaikes information criterion (AIC) and the conditional coefficient of determination (R^2) for the final variable sets are additionally given.

Response	**Model coefficients**	**Estimates**	***p*-value**	**SD**	**AIC**	**R^2**
Number of trapped *Culicoides*	colour: white	-16.2 ± 5.4	< 0.01		344.7	0.3
	pat (random effect)			9.3		
Number of trapped *C. chiopterus*	colour: white	-10.0 ± 3.8	< 0.05		312.3	0.2
	shape: quadratic	5.1 ± 3.7				
	pat (random effect)			3.1		
Number of trapped *C. dewulfi*	intercept	-1.36 ± 0.3	< 0.001		196.5	0.6
	pat (random effect)			8.4		

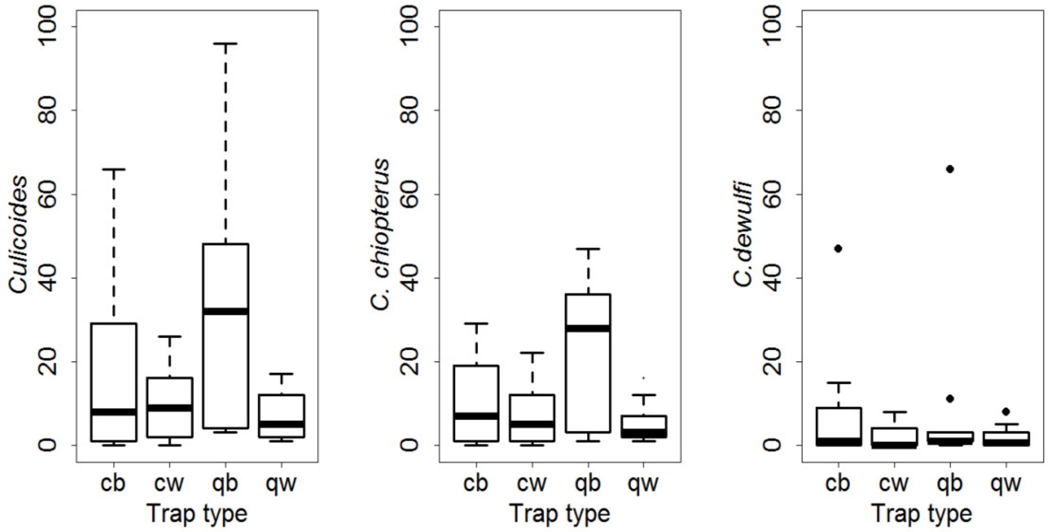

Figure 3.5 Number of trapped *Culicoides*, *C. chiopterus* and *C. dewulfi* specimens with cone-shaped black traps (cb), cone-shaped white traps (cw), quadratic black traps (qb) and quadratic white traps (qw). The boxplots describe the median (central bar) and the 25% and 75% quartiles (upper and lower boundary of the box, respectively). The whiskers extend to the lowest and highest data points, that are still within 1.5x the interquartile range (= length of the box).

Table 3.5 Minimal (min), mean (± standard deviation) and maximal (max) relative humidity (%) and temperature (°C) measured within the four trap types within pats (pat), outside of pats (air) and outside of traps (ambient, only temperature, recorded from the nearby weather station of the University of Oldenburg, www.uni-oldenburg.de/wetter). In addition, the mean light intensity (lx) (± standard deviation) measured within the trap bodies is given.

		Temperature [°C]			**Relative humidity [%]**			**Light intensity [lx]**
		min	mean	max	min	mean	max	mean
Ambient		9.4	18.6 ± 4.3	31.3	24.1	66.2 ± 18.8	96.1	
Cone-black	Air	7.2	23.5 ± 10.4	57.1	20.9	78.5 ± 21.3	100	5500 ± 6189
	Pat	9.7	21.9 ± 6.7	42.9				
Cone-white	Air	7.1	23.7 ± 10.8	59.3	9.4	74.6 ± 22.8	100	20250 ± 4119
	Pat	9.2	23.0 ± 7.9	52.7				
Quadratic-black	Air	7.3	22.8 ± 9.4	52.9	24.7	76.8 ± 18.8	100	4180 ± 901
	Pat	8.8	21.8 ± 6.7	47.3				
Quadratic-white	Air	7.4	22.5 ± 9.3	51.8	19.9	77.9 ± 18.8	99.7	14140 ± 1704
	Pat	8.8	21.9 ± 6.9	45.6				

3.4 DISCUSSION

Various methods are available to determine the density of immature stages of *Culicoides* in breeding substrates, but information on the relative effectiveness of these methods is limited. Kline et al. (1975) compared sieve-, sand- and magnesium sulphate-flotation with Berlese funnel-extraction and rearing chambers and obtained most *Culicoides* specimens with sieve- and salt-flotation. Instead, Campbell and Kettle (1976) obtained fewer individuals of the dung-breeding species *C. brevitarsis* by magnesium sulphate-flotation compared to emergence traps. Also the present comparison of the performance of emergence traps, sugar-flotation and Berlese demonstrated that the effectiveness of these methods differed significantly – emergence traps sampled *Culicoides* less successfully in comparison to the extraction techniques. There are several potential explanations for this result: possibly, more *Culicoides* emerged without being trapped in the top collection container. No accumulation of dead midges was observed inside the traps, but dried-up individuals of insects of such a small size as *Culicoides* are probably hardly visible on the substrate after more than six weeks. Moreover, while the numbers of successfully emerged individuals in traps might have been influenced by a natural mortality of the immature stages during development, this is without influence on the number of larvae obtained with the extraction methods.

Although the present sampling design aimed at overwintering *Culicoides* larvae, hibernation processes are unlikely related to the lower individual numbers in emergence traps. The rearing success from larvae extracted with the Berlese was very high and the larvae were active immediately after extraction. Larvae of the primary vector of BTV in North America, *C. variipennis*, showed a similar behaviour upon collection from a frozen pond mud, being active right after thawing (Vaughan and Turner, 1987; reviewed by Tabachnick, 1996).

The authors of a previous study concluded that flotation underestimates the total colonisation density of all developmental stages, as *Culicoides* eggs are too small to be recovered (Campbell and Kettle, 1976). While this does probably not apply to the present study, dealing with overwintering larvae, it has to be considered when different developmental stages are to be expected in the studied substrate. Thus, the same experiment during summer might have resulted in higher individual numbers in emergence traps, provided that eggs or younger larvae are given enough time to complete development. This assumption is, however, not supported by the results of Kline et al. (1975), who did conduct their study during summer. Moreover, they found the Tullgren-funnel to be the least successful method in comparison to several flotation techniques, pointing out that the affectivity of a method can differ among species and breeding substrates.

Generally, all methods tested were quite applicable to cow dung. The somewhat higher number of larvae obtained with sugar-flotation was not significantly different from that obtained with Berlese. However, flotation proved to be much more labour-intensive and the results obtained are likely to be affected by the experience of the operator and the time spent on the examination of a sample. Instead, using Berlese, large numbers of samples can be surveyed in a comparably short time and the extraction process can be easily standardised for light intensity, sample amount and processing time. This method was highly appropriate in order to extract *Culicoides* larvae from cowpats. Moreover, the Berlese provides the possibility to test potential breeding substrates, e.g., in advance to experimental studies in order to avoid working with samples without immature *Culicoides*. A further advantage of the Berlese technique is the good viability of extracted larvae collected in tap water, which allows highly successful rearing to adults. Individuals of the Obsoletus complex have also been reared after being extracted from dungheaps with sugar-flotation (see Manuscript VI, section 11.2.1), but the rearing success was low (21%) compared to the results we obtained with larvae extracted via Berlese.

It can be concluded that the extracted larvae were in the fourth instar as the length and width of the head capsules closely resembled the measurements of Kettle and Lawson (1952) for fourth instar individuals (Table 3.3). For the purposes of this study, the presence of different life stages would have been of disadvantage for an accurate evaluation. Eggs or very young instars could have increased the number of *Culicoides* captured with emergence traps, which are immobile (eggs) or too small to be recovered with Berlese or flotation. Consequently, the results merely apply to later larval instars and provide no information on the effectiveness for other life stages.

Despite the comparably low numbers of *Culicoides* obtained with emergence traps, this tool is among the most significant tools for breeding site studies of *Culicoides*. Therefore, knowledge that can potentially help to improve the effectivity of emergence traps is particularly important. The present study provided no evidence that the trap shape significantly affected the trapping success. Instead, the comparison of black and white traps revealed that a reduced light intensity within the trap body, i.e., a stronger contrast between trap and collection container, resulted in a higher trapping success of dung-breeding *Culicoides*. These results are most likely due to the positive phototaxis of *Culicoides*: assuming comparable numbers of individuals emerged from subsamples in differently coloured traps, fewer individuals have reached the collection container at the top of the white traps. These significant differences encourage the discussion of the first study: the low individual numbers in emergence traps compared with the extraction methods might account to the fact that not all emerged midges were actually trapped.

In light of these results, an adequate trap design should include a non-transparent trap body to maximise the differences in light intensity between trap and collection container. In this context, an important issue is the risk of a heat-up within the traps. The moderate heat-up in emergence traps in the present study, did not differ considerably between trap types. The cooling effect by evaporation of the saline and from cowpats in combination with the ventilation through the aeration windows presumably compensated for potential overheating. Nevertheless, especially in warm-climate regions, this risk should be minimised. For *C. brevitarsis*, Allingham (1991) found temperatures above 35.5 °C impeding a successful development of immature stages. In the present study, temperature within all trap types sometimes exceeded this value (Table 3.5). Thus, the trapping success in the present study might have been higher at overall lower temperatures. An alternative are trap bodies made from white gauze as proposed by Pajor (1987). However, the choice of design appears to imply a trade-off between a higher trapping success by using a non-transparent trap body and reducing the risk for a heat-up by applying an air-permeable material. Overall, an opaque trap material with sufficient aeration is recommendable in order to maximise the trapping success. Prospective studies might consider traps constructed of a reflecting material. Ideally, an optimised design should combine both, a maximised light gradient between the trap body and the collection container along with sufficient permeability of the used material.

CHAPTER 4

BREEDING HABITATS OF THE OBSOLETUS GROUP

Chapter 4 addresses one major question: where do the species of the Obsoletus Group develop? A comprehensive field study, investigating potential breeding habitats of *C. obsoletus* s.s., *C. scoticus*, *C. chiopterus* and *C. dewulfi* on cattle farms throughout Germany, aimed to answer this question. Moreover, in a preceding literature review of chapter 4, the prior state of knowledge regarding the breeding sites of these species is summarised in order to support the evaluation of different habitat types.

This chapter is based on results published by the journal **Medical and Veterinary Entomology** [→ manuscript III (*Emergence of Culicoides obsoletus group species from farm-associated habitats in Germany*), section 11.1.3].

4.1 INTRODUCTION

Generally, the knowledge on breeding site preferences of *Culicoides* is extremely poor. This is also true for the species of the Obsoletus Group. However, a better understanding of the breeding ecology of veterinary relevant species is crucial for the development and application of control measures. In this context, the first basic step is the identification of important breeding sites, in order to possibly allow their modification or removal. Preventing the development and emergence of *Culicoides* will effectively suppress the adult population.

This study investigated cattle farms as livestock holdings represent an environment where potential vectors and hosts coincide with suitable breeding substrates. Recently, studies from Spain, South-East England, Northern Ireland and Belgium supplied information on potential breeding sites in rural surroundings (González et al., 2013; Harrup et al., 2013; Thompson et al., 2013; Zimmer et al., 2013). The present study presents a comprehensive survey on cattle farms throughout Germany in order to provide a more detailed and substantial knowledge on the significance of different types of breeding habitats. This assessment is supported by a literature review summarising available information on breeding sites of the Obsoletus Group.

4.1.1 LITERATURE REVIEW OF DEVELOPMENTAL SITES

The following sections summarise and discuss available information on breeding sites for each species of the Obsoletus Group separately. The records were classified in order to facilitate an appraisal of the relevance of different habitat types, e.g., "dung" or "plant material". However, in many cases, the provided information on habitats in the literature was limited, impeding the assignment to a class. Therefore, the original descriptions of the breeding sites are always given in addition.

In the species tables the cited references are represented by numbers and the according references are listed in Table 4.1. Differences regarding sampling techniques often impede a meaningful comparison of the results. Nevertheless, the review includes the number of samples to give an impression on the abundances in breeding substrates (e.g., were specimens collected from one cowpat or 500 fungus fruit bodies). Studies that did not differentiate between the species, i.e., refer to data on the species group level, were not included.

4.1.1.1 *C. OBSOLETUS* S.S.

A large number of records on breeding sites, scattered throughout the literature, can be found for the eponym species of the Obsoletus Group (Table 4.2). The reported habitats are very diverse, including dung, different types of decaying plant material, the margins of water bodies, bogs or forest soil. A large number of the 53 records (45%) are associated with the agricultural environment, particularly with animal dung (37%). This habitat class also produces comparably large number of specimens. *C. obsoletus* s.s. occurred in the dung of cattle, sheep, horses and chicken. Thus, the animal species does not seem to be a decisive factor. Possibly, the age of the dung or the state of decomposition, respectively, plays a role. Notably large numbers of individuals were obtained from "old and composted manure" (no. 4, González et al., 2013) or "old litter in the angles of cattle stables" (no. 6, Ninio et al., 2011b). However, large numbers of individuals also emerged from "fresh" sheep dung (no. 10, González et al., 2013). While dungheaps appear to be productive sites for *C. obsoletus* s.s., only two studies report the occurrence of this species in cowpats (no. 9, Zimmer et al., 2014; no. 11, Hanski, 1980). According to the literature review, also *C. scoticus* rarely occurs in this habitat (see section 4.1.1.2).

The records classified as "vegetation" provide rather unspecific information, as the breeding sites described by Harrup et al. (2013) refer to categories pre-defined by the Joint Nature Conservation Committee. It remains unknown if individuals emerged from the vegetation, decaying plant material, ground substrate or else. A considerable number of breeding sites mentioned in other studies refer to plant material (26% or 36% if silage residues and vegetation are included) without indications that the plant species matters. *Culicoides obsoletus* s.s. was sampled from sugar beet residues, garden compost, cornstalks, spruce needles and fungi and repeatedly from leaf litter. According to comprehensive surveys on Diptera breeding in fungi, this species did not emerge from this type of habitat (Hackman and Meinander 1979; Sevčík 2006).

In four studies, *C. obsoletus* s.s. developed in material taken from tree-holes. Presumably, the decaying plant material within this habitat plays a greater role as the tree hole itself. Water-filled tree-holes were not mentioned, whereas several authors report the occurrence of *C. obsoletus* s.s. in substrates associated with water bodies. These records mostly refer to the margins or banks of rivers. In the case of the highland brooks, investigated by Havelka (1976, no. 49), it cannot be confined from where exactly *C. obsoletus* s.s. emerged. In this study, large "greenhouses", built over sections of the brooks, covered hydrous areas and banks alike. The only waterlogged habitat mentioned beyond, was a "flooded meadow" (no. 50, Zimmer et al., 2013b). Overall, water bodies or associated substrates do not appear to be relevant as breeding sites for *C. obsoletus* s.s..

Table 4.1 List of references mentioning or describing breeding sites of the species of the Obsoletus Group. The assigned reference numbers (1 – 29) represent these cited studies in the following species tables (Table 4.2 – 4.5).

Ref.-no.	**Reference**
1	Goetghebuer (1936 cited by Harrup, 2014)
2	Edwards (1939)
3	Hill (1947)
4	Kettle and Lawson (1952)
5	Murray (1957)
6	Buxton (1960)
7	Campbell and Pelham-Clinton (1960)
8	Laurence (1953)
9	Kettle (1962)
10	Weinburgh and Pratt (1962 cited by Zimmer et al., 2013b)
11	Hanski (1980)
12	Jamnback (1965)
13	Kremer (1965)
14	Braverman et al. (1974)
15	Havelka (1976)
16	Hackman and Meinander (1979)
17	Boorman (1986)
18	Froese and Havelka (1991)
19	Dely-Draskovits and Babos (1993 cited by Krivosheina, 2008)
20	Hövemeyer and Havelka (1996)
21	Drissner et al. (1994)
22	Sevčík (2006)
23	Zimmer et al. (2010)
24	Ninio et al. (2011b)
25	González et al. (2013)
26	Harrup et al. (2013)
27	Zimmer et al. (2013a)
28	Zimmer et al. (2013b)
29	Zimmer et al. (2014)

Table 4.2 Breeding sites of *C. obsoletus* s.s described in the literature. The number of individuals (IND) from the according number of samples investigated (n) is given along with the applied methods for sampling (SM) and the country of data collection (C, abbreviations following the International Organization for Standardization (ISO-3166)) and cited reference (R, see Table 4.1). In column "+ *C. s.*" it is stated if *C. obsoletus* s.s. was found in association with *C. scoticus* (1 = in association, 0 = not in association).

No.	**Class**	**Breeding site**	**IND**	**n**	**+ *C.s.***	**SM**	**C**	**R**
1	dungheap (cattle)	muck heaps	175 (♂+♀)*^{1}	15	0	EF	UK	26
2	dungheap (cattle)	cattle manure heap	335 (♂+♀)	5	0	EL	FR	24
3	dungheap (cattle)	pile of mixed bedding and cow manure	n.a.	n.a.	0	FEL, B	USA	12
4	dungheap (sheep)	old and composted manure	4790 (♂+♀)	24	1	EF	ES	25
5	dung (cattle)	dried cowdung inside a cowshed	52 (♂)	15	0	EL	BE	23
6	dung (cattle)	old litter in the angles of cattle stables	1589 (♂+♀)	5	0	EL	FR	24
7	dung (cattle)	dung adhering to walls inside cowshed	64 (♂)	28	0	EL	BE	29
8	cowpats	cow droppings	n.a.	n.a.	0	F	FI	11
9	cowpats	cow dung in pasture	1 (♂)	241	0	EL	BE	29
10	dung (sheep)	fresh manure	845 (♂+♀)	24	0	EF	ES	25
11	dung (sheep)	farm corner (mixture of manure and moist organic matter)	2219 (♂+♀)	24	1	EF	ES	25
12	dung (sheep)	sheep dung in fields	n.a.	n.a.	0	n.a.	UK	2
13	dung (horse)	horse dung	n.a.	n.a.	0	EF	UK	7
14	dung (chicken)/soil	components of chicken coop	70 (♂)	28	0	EL	BE	29
15	dung	middens	n.a.	n.a.	0	EF	UK	7
16	soil (livestock)	substrate surrounding dungheaps	95 (♂+♀)*^{1}	12	1	EF	UK	26
17	soil (livestock)	soil of a livestock trampling area	2 (♂)	66	0	EL	BE	29
18	soil (livestock)	soil polluted with chicken or horse manure	n.a.	n.a.	0	FEL, B	USA	12
19	soil (arable land)	arable land	1 (♂+♀)	5	0	EF	DE	18
20	soil (clayey)	marshy area with clayey soil	279 (♂+♀)	n.a.	0	S	UK	3
21	soil (salty)	salty terrain	n.a.	n.a.	0	n.a.	FR	13
22	soil (forest)	forest mud (flooded muddy soil with a humus layer in the forest)	4 (♂+♀)	24	0	EF	ES	25
23	soil	wet soil rich in organic matter	n.a.	n.a.	0	F, ET	IL	14
24	soil (livestock)	grazed orchard	n.a.	n.a.	0	ET	DE	21
25	cattle food	leftover food along the feed bunk	32 (♂)	60	0	EL	BE	29
26	silage	silage residues	90 (♂)	88	n.a.	EL	BE	29
27	silage	silage (maize, grass and sugar beet pulp)	184 (♂)	77	1	EL	BE	27
28	silage	maize silage residues	311 (♂+♀)*^{2}	2	n.a.	ELI	BE	28
29	vegetation/ waterbody	marginal vegetation surrounding open water	75 (♂+♀)*^{1}	8	1	EF	UK	26
30	vegetation	broadleaved woodland vegetation	5 (♂+♀)*^{1}	25	1	EF	UK	26
31	fungi	dry decaying fungi	n.a.	n.a.	0	n.a.	UK	2

32	plant material	broadleaved woodland leaf litter	120 (♂+♀)*^{1}	13	0	EF	UK	26
33	plant material	shaded compost heap of damp leaves	< 20 (♂+♀)	n.a.	0	EF	USA	5
34	plant material	ditch with rotting leaves	n.a.	n.a.	0	S	UK	3
35	plant material	decaying leaves under a hedge bank	n.a.	n.a.	0	S	UK	3
36	plant material	compost pile of sugar beet residues	4 (♂)	4	1	EL	BE	29
37	plant material	garden compost heap	n.a.	n.a.	0	EF	UK	7
38	plant material	moist straw	n.a.	n.a.	0	FEL, B	USA	12
39	plant material	decaying spruce needles mixed with twigs and wood chips	n.a.	n.a.	0	FEL, B	USA	12
40	plant material	decaying cornstalks	n.a.	n.a.	0	FEL, B	USA	12
41	treehole	treehole containing decaying leaves	n.a.	n.a.	0	S	UK	3
42	treehole	tree holes rich in decaying matter	7 (♂+♀)	n.a.	0	EF	USA	5
43	treehole	damp debris from a treehole	n.a.	n.a.	0	n.a.	UK	2
44	treehole	treeholes of beech trees	n.a.	n.a.	0	n.a.	FR	13
45	waterbody margin	sandy stream banks devoid of humus	< 20 (♂+♀)	n.a.	0	EF	USA	5
46	waterbody margin	muddy edges of springs	n.a.	n.a.	0	n.a.	DE	2
47	waterbody margin	riverbank in forest with herb layer	n.a.	n.a.	0	EF	DE	20
48	waterbody margin	damp sand surrounding a river	2 (♂+♀)	24	1	EF	ES	25
49	waterbody/ waterbody margin	two highland brooks	43 (♂+♀)	n.a.	0	EF	DE	15
50	waterbody	ground of a flooded meadow	1 (♂+♀)	2	n.a.	ELI	BE	28
51	waterbody	silt from a pond out of water	1 (♂+♀)	2	n.a.	ELI	BE	28
52	bogland/ marshland	marshland	n.a.	n.a.	0	F	UK	4
53	roof runoff	roof runoff (area with grass just below the barn roof)	2 (♂+♀)	24	1	EF	ES	25

F = flotation, EF = emergence in the field (emergence trap), EL = emergence in the laboratory, ELI = emergence in the laboratory following incubation, S = sieving, B = Berlese, *^{1} Numbers estimated accord. to figure, *^{2} Obsoletus complex, n.a. information not available.

4.1.1.2 *C. SCOTICUS*

The total number of 25 records of *C. scoticus* breeding sites found in the literature (Table 4.3) is not as low as might be expected considering the statements of other researchers (e.g., Harrup et al., 2013; Zimmer et al., 2013b). However, the species was not found as often as C. obsoletus s.s.. Possibly, the difficulties in differentiating the females of both species (see section 2.3) contribute to this result. Particularly older publications, based on the morphological identification of females only, may be prone to error. The fact that these species are often found in association (e.g., in 46% of references referring to *C. scoticus*) adds to this uncertainty.

In contrast to *C. obsoletus* s.s., animal dung seems to be a subordinate breeding habitat for *C. scoticus* (12% of reported cases) as compared to *C. obsoletus* s.s.. Few specimens were obtained from the dung of sheep and from cowpats. Instead, a certain significance of fungi as a breeding habitat needs to be considered. Four studies, investigating the sporophores of macrofungi, reported emergence of *C. scoticus* from different fungal species. However, in light of the large numbers of fungus samples processed in these studies (up to 3700 fruit bodies), the number of obtained individuals appears rather small (nos. 11 - 14, Hackmann and Meinander, 1979; Dely-Draskovits and Babos, 1993 cited by Krivosheina, 2008; Sevčík, 2006). Moreover, the sporophores of many fungi are restricted to short vegetation periods and decompose rapidly (Buxton, 1960), limiting their potential as a constant source for *Culicoides*. Nevertheless, macrofungi appear a suitable breeding substrate for *C. scoticus.*

Despite the unexpected large number of records, only limited numbers of individual occurred in most of the studies. One exception are the results of González et al. (2013, no. 16), who sampled 584 individuals from decaying leaves. The observed association with the parasitic flower plant *Lathraea clandestina* L. might be coincidental, as also discussed by the authors. Presumably, the presence of decaying leaves or other decaying plant material plays a more important role. Overall, including fungi, 36% of the breeding sites found in the literature refer to plant material, but – apart from the observation of González et al. (2013) – those breeding substrates were rarely very productive.

Table 4.3 Breeding sites of *C. scoticus* described in the literature. The number of individuals (IND) from the according number of samples investigated (n) is given along with the applied methods for sampling (SM) and the country of data collection (C, abbreviations following the International Organization for Standardization (ISO-3166)) and cited reference (R, see Table 4.1). In column "+ *C.o.*" it is stated if *C. scoticus* was found in association with *C. obsoletus* s.s. (1 = in association, 0 = not in association).

No.	Class	Breeding site	IND	n	+ *C.o.*	SM	C	R
1	dungheap (sheep)	old and composted manure	17 (♂+♀)	24	1	EF	ES	24
2	dung (sheep)	farm corner (mixture of manure and moist organic matter)	3 (♂+♀)	24	1	EF	ES	24
3	cowpat	cow dung in pasture	2 (♂)	241	0	EL	BE	28
4	soil (livestock)	substrate surrounding muck heaps	7 (♂+♀)*^{1}	12	1	EF	UK	25
5	soil (forest)	mud of a wheel-track in the wood	n.a.	n.a.	0	EL	FR	25
6	soil (forest)	forest soil with herb layer	n.a.	n.a.	0	EF	DE	20
7	silage	maize silage residues	311 (♂+♀)*^{2}	2	n.a.	ELI	BE	27
8	silage	silage (maize, grass, sugar beet pulp)	3 (♂)	77	1	EL	BE	26
9	silage	silage residues	2 (♂)	88	n.a.	EL	BE	28
10	vegetation/ waterbody margin	marginal vegetation surrounding open water	1 (♂+♀)*^{1}	8	1	EF	UK	25
11	fungi	7 different fungal species	42 (♂)	n.a.	0	EL	UK	6
12	fungi	17 different fungal species	94 (♂+♀)	580	0	EL	CZ, SK	22
13	fungi	3 different fungal species	25 (♂+♀)	3700	0	EL	FIN	16
14	fungi	different fungal species	n.a.	n.a.	n.a.	n.a.	HUN	19
15	plant material	broadleaved woodland leaf litter	20 (♂+♀)*^{1}	13	1	EF	UK	25
16	plant material	fallen leaves (moist decaying leaves, frequently utilized by *Lathraea clandestina*)	584 (♂+♀)	24	0	EF	ES	24
17	plant material/ soil	poplar grove soil (grass with fallen leaves, which usually remained inundated)	11 (♂+♀)	24	0	EF	ES	24
18	plant material	compost pile of sugar beet residues	1 (♂)	4	1	EL	BE	28
19	plant material	pine litter	1 (♂)	1	0	EL	UK	8
20	waterbody margin	river edges (damp sand surrounding the river with sandstone structure)	2 (♂+♀)	24	1	EF	ES	24
21	waterbody margin	pond (vegetated area)	2 (♂+♀)	24	0	EF	ES	24
22	waterbody	ground of a flooded meadow	1 (♂+♀)*^{2}	2	1	ELI	BE	27
23	waterbody	silt from a pond out of water	1 (♂+♀)*^{2}	2	n.a.	ELI	BE	27
24	marshland	marshy areas	n.a.	n.a.	n.a.	EF	UK	17
25	roof runoff	roof runoff (area with grass just below the barn roof)	4 (♂+♀)	24	1	EF	ES	24

EF = emergence in the field (emergence trap), EL = emergence in the laboratory, ELI = Emergence in the laboratory following incubation, S = sieving, B = Berlese, *^{1} Numbers estimated accord. to figure, *^{2} Obsoletus complex, n.a. information not available

4.1.1.3 *C. CHIOPTERUS* AND *C. DEWULFI*

Largest individual numbers of *C. chiopterus* and *C. dewulfi* were reported from cowpats or the soil underneath (26% in *C. chiopterus* and 33% in *C. dewulfi*, Tables 4.4 and 4.5). Kettle and Lawson (1952), who investigated a wide range of breeding substrates, highlighted that they exclusively found these species in "cow dung left lying naturally in the field", i.e., cowpats (no. 1 in both Tables). Most likely, also Campbell and Pelham-Clinton (1960) refer to cowpats when mentioning cattle dung (no. 6 in both Tables).

The two species were often found in association, i.e., in 39% of breeding sites reported for *C. chiopterus* and 50% of records for *C. dewulfi*. Apart from cattle dung, other habitats seem to be colonised occasionally in low density. Single specimens of both species occurred in silage, pine litter or the margins of water bodies. Moreover, *C. chiopterus* emerged from fungi and cattle food residues. Thus, even though it appears that cowpats are the most important breeding habitat, *C. chiopterus* and *C. dewulfi* do not exclusively develop in this substrate, as other researchers previously concluded (Dijkstra et al., 2008).

Table 4.4 Breeding sites of *C. chiopterus* described in the literature. The number of individuals (IND) from the according number of samples investigated (n) is given along with the applied methods for sampling (SM) and the country of data collection (C, abbreviations following the International Organization for Standardization (ISO-3166)) and cited reference (R, see Table 4.1). In column "+ *C.d.*" it is stated if *C. chiopterus* found in association with *C. dewulfi* (1 = in association, 0 = not in association).

No.	**Class**	**Breeding site**	**IND**	**n**	**+ *C.d.***	**SM**	**C**	**R**
1	cowpat	cowdung left lying naturally in the field	252 (♂+♀)^{*1}	3	1	F	UK	4
2	cowpat	cowpats on humid ground	n.a.	n.a.	n.a.	n.a.	FR	13
3	cowpat	cowdung located in pastures	"large numbers"	241	1	EL	BE	29
4	cowpat	old cattle dung in the meadow	133 (♂+♀)	2	1	ELI	BE	28
5	cowpat	cattle dung lying naturally in the field	n.a.	n.a.	n.a.	n.a.	UK	9
6	dung (cattle)	cowdung	n.a.	n.a.	1	EF	UK	7
7	dung (horse)	horse dung lying naturally in the field	n.a.	n.a.	n.a.	n.a.	UK	9
8	cattle food	leftover food along the feed bunk	n.a.	n.a.	0	EL	BE	29
9	silage	maize silage residues	1 (♂+♀)	2	1	ELI	BE	28
10	soil (silage)	wet soil between silage reserves	n.a.	n.a.	0	EL	BE	29
11	soil (livestock)	soil of a livestock trampling area	n.a.	n.a.	0	EL	BE	29
12	soil (livestock)	soil under cowdung	"large numbers"	60	1	EL	BE	29
13	soil (livestock)	moist soil polluted with chicken manure	n.a.	n.a.	0	FEL,B	USA	12
14	soil (livestock)	ground under the old cattle dung	23 (♂+♀)	2	1	ELI	BE	28
15	soil (livestock)	ground of an area affected by cattle	1 (♂+♀)	2	0	ELI	BE	28
16	soil (livestock)	grazed orchard	n.a.	n.a.	0	ET	DE	22
17	fungi	two different species of fungi	3 (♂+♀)	n.a.	0	EL	FIN	16
18	plant material	pine litter	1 (♂)	1	1	EL	UK	8
19	plant material	moist straw	n.a.	n.a.	0	FEL,B	USA	12
20	elm sap	sap running from wounds of elm trees	n.a.	n.a.	0	n.a.	UK	2
21	waterbody margin	riverbank in forest with herb layer	n.a.	n.a.	1	EF	DE	20
22	waterbody/ pasture	ground of a flooded meadow	1 (♂+♀)	2	0	ELI	BE	28
23	bog/ plant material	bogs rich in decaying vegetation	n.a.	n.a.	0	n.a.	BE	1

EF = emergence in the field (emergence trap), EL = emergence in the laboratory, ELI = Emergence in the laboratory following incubation, S = sieving, B = Berlese, ^{*1} including *C. dewulfi,* n.a. information not available.

Table 4.5 Breeding sites of *C. dewulfi* described in the literature. The number of individuals (IND) from the according number of samples investigated (n) is given along with the applied methods for sampling (SM) and the country of data collection (C, abbreviations following the International Organization for Standardization (ISO-3166)) and cited reference (R, see Table 4.1). In column "+ *C. ch.*" it is stated if *C. dewulfi* was found in association with *C. chiopterus* (1 = in association, 0 = not in association).

No.	**Class**	**Breeding site**	**IND**	**n**	**+ *C. ch.***	**SM**	**C**	**R**
1	cowpats	cow dung left lying naturally in the field	252 (♂+♀)[*1]	3	1	F	UK	4
2	cowpats	cowpats, collected from acid soil	n.a.	n.a.	n.a.	n.a.	FR	13
3	cowpats	cowdung located in pastures	"large numbers"	241	1	EL	BE	28
4	cowpats	old cattle dung in the meadow	82 (♂+♀)	2	1	ELI	BE	27
5	cowpats	cattle dung lying naturally in the field	n.a.	n.a.	n.a.	n.a.	UK	9
6	dung (cattle)	cowdung	n.a.	n.a.	1	EF	UK	7
7	dungheap	manure piles	n.a.	n.a.	n.a.	n.a.	n.a.	10
8	dung (horse)	horse dung lying naturally in the field	n.a.	n.a.	n.a.	n.a.	UK	9
9	dung/straw	straw contaminated with faeces	n.a.	n.a.	n.a.	n.a.	n.a.	10
10	soil (livestock)	soil under cowdung	"large numbers"	60	1	EL	BE	28
11	soil (livestock)	ground under the old cattle dung	19 (♂+♀)	2	1	ELI	BE	27
12	soil (forest)	forest soil with herb layer	n.a.	n.a.	0	EF	DE	20
13	soil	molehill soil on pasture	< 6 (♂+♀)	36	0	EL	BE	28
14	silage	silage (maize, grass, sugar beet pulp)	1 (♂+♀)	77	0	EL	BE	26
15	silage	maize silage residues	8 (♂+♀)	2	1	ELI	BE	27
16	plant material	pine litter	5 (♂)	1	1	EL	UK	8
17	waterbody margin	riverbank in forest with herb layer	n.a.	n.a.	1	EF	DE	20
18	waterbody/ waterbody margin	highland brooks	13 (♂+♀)	n.a.	0	EF	DE	15

F = Flotation, EF = emergence in the field (emergence trap), EL = emergence in the laboratory, ELI = Emergence in the laboratory following incubation, S = sieving, B = Berlese, [*1] including *C. chiopterus,* n.a. information not available.

4.1.1.4 CONCLUSIONS

Overall, the literature review underlines that livestock holdings offer suitable breeding substrates for all species of the Obsoletus Group. Records of "natural" breeding sites are rare (e.g., Havelka 1976; Murray, 1957). However, it has to be considered that this is mainly accounted to the focus on the investigation of sheep- and cattle holdings in consequence of the emergence of BTV (e.g., González et al., 2013; Harrup et al., 2013; Zimmer et al., 2013a), whereas only a limited number of studies analysed natural habitats. Most information is available for breeding sites of *C. obsoletus* s.s.. Considering the diversity of colonised substrates, this species appears to be quite opportunistic in the choice of development sites. However, animal dung in form of dungheaps or dung litter seems to play a major role. Instead, no explicitly important breeding habitat could be curtailed for *C. scoticus*. Fungi and other decaying plant material seem to provide suitable breeding substrates. Apart from that, the diversity of records almost equals that of *C. obsoletus* s.s.. For *C. chiopterus* and *C. dewulfi*, cowpats appear be a significant breeding habitat, although these species cannot be classified as exclusive dung breeders.

The majority of references provide information from European countries, the year of publication ranging from 1939 to 2014. In most studies individuals were collected with emergence traps in the field (EF, 41%), or substrate was transferred to the laboratory to allow the emergence of *Culicoides* in a more controlled environment (EL, 41%), often combined with incubation of the material in order to accelerate the development of immatures. Fewer studies obtained immature stages via flotation methods (14%) or sieving (5%).

4.2 FIELD STUDY: EMERGENCE OF OBSOLETUS GROUP SPECIES FROM FARM-ASSOCIATED HABITATS IN GERMANY

4.2.1 INTRODUCTION

The field study presented in this chapter investigated potential breeding habitats of the Obsoletus Group species in agricultural environments. Twenty cattle farms, distributed throughout Germany, were surveyed to analyse possible habitat preferences and relations between the abundance of *Culicoides* and physico-chemical characteristics of habitats. However, the latter aspect will be the focus of chapter 5. The breeding sites primarily surveyed in the present investigation comprised dungheaps, cowpats, compost, bark mulch (usually on flower beds) and habitats associated with water bodies. The preceding literature review on breeding sites (see section 4.1.1) pointed at the importance of animal dung for most species of the Obsoletus Group. Instead, substrates associated with water bodies did not appear to be among the relevant sources of any of the four species. Nevertheless, this habitat type was included in the survey in order to verify this conclusion with regard to the agricultural environment. Moreover, species of the Pulicaris Group are expected to breed in waterlogged substrates or the vicinity of water bodies (Kettle and Lawson, 1952; Foxi and Delrio, 2010; Kirkeby et al., 2010). Compost heaps and bark mulch were included, as previous studies indicated these substrates to be among the breeding sites of the Obsoletus Group (Kiel et al., 2009).

4.2.2 METHODS

Potential breeding habitats (Figure 4.1) were investigated on 20 cattle farms, distributed throughout Germany (Figure 4.2) during four sampling periods: Aug. - Sept. 2012, Apr. - May 2013, June 2013 and July - Aug. 2013. One of the farms was replaced after the first sampling in 2012. During each period, up to 10 conical emergence traps (white-coloured, see section 3.2.2) per farm were set up on the potential breeding substrates for 14 consecutive days. A mixture of aqua dest., ethanol (96%) and glycerine (ratio 4:3:2), supplemented with a drop of detergents, served as a preservation liquid. As far as possible, the same habitat types were investigated on all farms in order to warrant comparability of the results achieved. Thus, the number of different habitat classes examined was reduced in favour of the collection of sufficient amounts of data.

A total of 229 traps were placed on dungheaps (total number of all traps on dungheaps, at all farms and during all sampling periods). Further habitat types investigated were cowpats (225 traps), water-associated substrates (241 traps), bark mulch (23 traps) and compost (11 traps). During April and May 2013, mainly cowpats from the previous year were investigated in order to target the overwintering *Culicoides* population. The trapping of specimens from water-associated substrates usually took place upon the moist sediment close to the waterline, the bank or the centre, if drying up of the water bodies had occurred. This habitat type comprised ditches (90 traps), ponds (74 traps), puddles (31 traps), leakage from silage, dungheaps or slurry (22 traps), reed filtration systems (11 traps), rain drainage (7 traps) and boggy grass areas (6 traps).

Trapped insects were removed after the sampling period of 14 days. Biting midges of the genus *Culicoides* were preserved in ethanol (70%) for subsequent morphological identification of the females of the Obsoletus Group, following the key introduced in chapter 2 (see section 2.4). Males were identified according to the key of Campbell and Pelham-Clinton (1960). The individual numbers of *C. chiopterus* and *C. dewulfi* that emerged from cowpats (excluding samples with zero observations) were compared using a Wilcoxon signed-rank test and the frequency of their presence using a two-sample proportions test (R Core Team, 2014).

Figure 4.1 Emergence traps on potential breeding habitats of the Obsoletus Group, i.e., on cowpats in the pasture (left) and on a dungheap (right).

Subsequent to the *Culicoides* collection, a substrate sample of on each site was taken for soil analysis in the laboratory (sample of the substrate under the emergence trap, down to a depth of 10 cm) in order to measure the concentration of potassium and phosphate, the pH value, the percentage of organic matter (the difference between dry weight and ash-free dry weight) and the ratio of carbon to nitrogen (CN-ratio) with a CHNS analyser (Flash 2000, Thermo Fisher Scientific, Braunschweig, Germany). In addition, the substrate moisture was measured on-site in soft or diluted substrates (e.g., dungheaps) with a moisture probe (HD2 in combination with a Trime®-Pico 64/32, Imko, Ettlingen, Germany). Where this method was not applicable (for firmer substrates or cowpats), three standardised substrate samples (150 cm^3 per sample) were taken instead (due to the small size, cowpats usually supplied enough material for one sample). These samples were weighed on site (fresh weight), and dried in the laboratory in order to determine the dry weight and water content (difference in weight between fresh and dry substrate).

The (potential) amount of shade at each sample site was estimated (never shaded, temporarily shaded, constantly shaded), taking shadow-casting objects in the vicinity of the traps into account. Also the height of the emergence traps on dungheaps was measured (distance from the bottom rim of the trap to the ground, ranging from zero to four metres). A portable GPS device (Garmin GPS Map 62S, Garching, Germany) determined the position of each trap. The analysis and results regarding the measured environmental parameters will be presented in chapter 5 (see section 5.2).

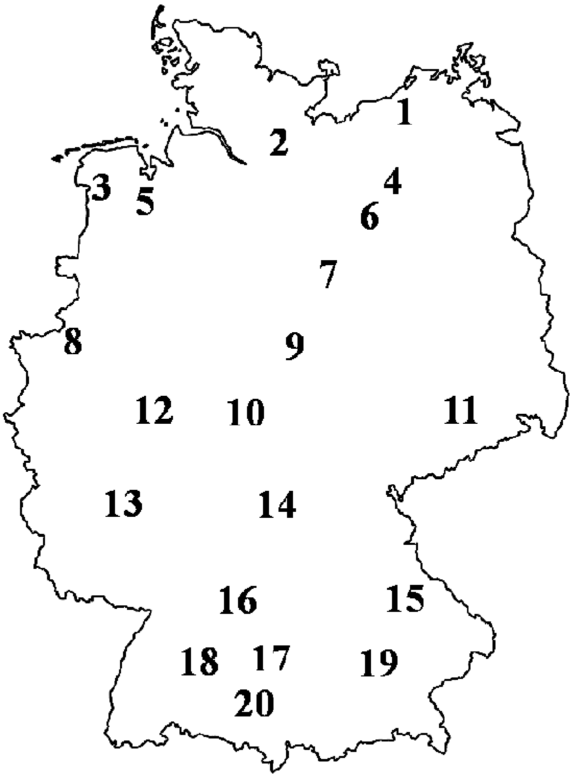

Figure 4.2 Locations of the 20 cattle farms in Germany. Farm 20 was included only in 2012 and farm 17 only in 2013.

4.2.3 RESULTS

A total of 24,564 individuals of the Obsoletus Group were collected with traps on **dungheaps** (11,693 males, 12,871 females). Of the male individuals, 11,533 were identified as *C. obsoletus* s.s. and two males as *C. scoticus.* Of the female *Culicoides* midges, 12,852 belonged to the Obsoletus complex. The extremely low number of male *C. scoticus*, lead to the conclusion that the vast majority of these 12,852 females were *C. obsoletus* s.s.. Small numbers of *C. chiopterus* (13 males, 9 females) and *C. dewulfi* (9 males) also occurred in dungheap samples (Table 4.6). The average number of emerged individuals from dungheaps at different farms varied widely (Table 4.7).

Table 4.6 Mean number (± standard deviation) of Obsoletus Group specimens in all samples (n) and positive samples (n (positive)) from dungheaps (DH), cowpats (CP), compost (CO), bark mulch (BM) and water-associated substrates (WB). Additionally, total numbers of individuals of single species that emerged from these habitats are given (n.d. = not determinable due to insufficient material conditions, e.g., missing hypopygium).

Habitat	**DH**	**CP**	**CO**	**BM**	**WB**
Obsoletus Group:					
Mean (all samples)	107.3 ± 465.1	14.2 ± 50.3	0.8 ± 1	1.9 ± 6.5	3.2 ± 37.0
Mean (positive samples)	221.3 ± 650.2	43.8 ± 80.9	1.8 ± 1	22.0 ± 7.1	59.3 ± 154.3
♂ Obsoletus Group	11,693	1,671	2	3	526
♀ Obsoletus Group	12,871	1,529	7	41	245
♂ *C. obsoletus* s.s.	11,533	28	1	2	455
♂ *C. scoticus*	2	432	0	0	64
♀ Obsoletus complex	12,852	231	7	41	245
♂ *C. chiopterus*	13	757	0	0	0
♀ *C. chiopterus*	9	795	0	0	0
♂ *C. dewulfi*	9	440	0	0	0
♀ *C. dewulfi*	0	502	0	0	0
♂ + ♀ n.d.	146	15	1	1	7
n	229	225	11	23	241
n (positive)	111	73	5	2	13

Table 4.7 Average number (mean ± standard deviation) of Obsoletus Group specimens sampled with emergence traps on dungheaps at the different farms (n = total number of samples from the dungheap of one farm). The results were arranged according to the mean number of emerged individuals.

Farm	**mean**	**n**	**Farm**	**mean**	**n**
Farm 11	0	9	Farm 14	44.4 ± 104.9	17
Farm 7	0.1 ± 0.5	14	Farm 10	61.3 ± 187.1	18
Farm 12	0.2 ± 0.4	12	Farm 16	72.0 ± 123.0	3
Farm 20	0.8 ± 1.5	4	Farm 19	96.5 ± 270.8	17
Farm 8	1.8 ± 2.1	4	Farm 18	113.0 ± 199.7	16
Farm 1	2.5 ± 6.2	10	Farm 17	183.7 ± 345.0	6
Farm 2	4.1 ± 9.8	12	Farm 3	185.4 ± 200.0	12
Farm 9	4.1 ± 9.9	20	Farm 13	329.2 ± 486.3	14
Farm 4	23.8 ± 58.1	9	Farm 15	605.5 ± 1466.4	18
Farm 5	24.6 ± 47.0	13	Farm 6	no dungheap	

A total of 3,200 individuals of the Obsoletus Group emerged from **cowpats**, mainly *C. chiopterus*. Moreover, 28 male *C. obsoletus* s.s., 432 male *C. scoticus* and 231 Obsoletus complex females were collected (Table 4.6). *Culicoides obsoletus* s.s. occurred in four samples and *C. scoticus* in 11 samples, while *C. chiopterus* emerged from 57 cowpats and *C. dewulfi* from 34. In 46.8% of cases, *C. chiopterus* and *C. dewulfi* were found in association. The first species occurred significantly more often than *C. dewulfi* (two-sample proportions test, $p < 0.01$) but not in significantly higher individual numbers (Wilcoxon signed-rank test, $p = 0.86$).

In five out of 11 samples from **compost heaps**, a total of nine *Culicoides* midges of the Obsoletus Group were found (two males, seven females, Table 4.6). One of the two males was identified as *C. obsoletus* s.s., the second could not be identified due to a missing hypopygium. On flower beds covered with **bark mulch**, 44 individuals of the Obsoletus Group were trapped. All females belonged to the Obsoletus complex. Two males were identified as *C. obsoletus* s.s. while the third male was not identifiable. Samples from **water-associated substrates** contained a total number of 771 specimens of the Obsoletus complex. Among male individuals, 455 were identified as *C. obsoletus* s.s. and 64 as *C. scoticus*. The 13 samples obtaining Obsoletus Group midges originated from diverse water bodies, such as ponds (five samples), ditches (four samples), slurry leakage (two samples), rain drainage (one sample) and puddles (one sample). Overall, low individual numbers (1.4 ± 0.9, mean ± standard deviation) were collected from the majority of these 13 samples. However, at a pond near to a dungheap, a total of 758 *Culicoides* midges (189.5 ± 250.1) emerged under four traps located on mud near the water edge and on a mat of floating grass. Only from a low number of breeding sites did species of the Pulicaris Group emerge (9% of all samples), i.e., *C. pulicaris* (Linnaeus) and *C. punctatus* (Meigen)).

Overall, the number of emerging *Culicoides* differed strongly among the four **sampling periods**. By far, the highest number of individuals of the Obsoletus Group emerged in April and May 2013, i.e., a total of 17,848 individuals were collected with emergence traps during that sampling period (Table 4.8). Least specimens were collected in July - Aug. 2013, i.e., 1,548 individuals. These differences primarily reflected the large number of *Culicoides* collected from dungheaps and cowpats.

Table 4.8 Number of Obsoletus Group individuals collected from dungheaps (DH), cowpats (CP), compost (CO), bark mulch (BM) and water-associated substrates (WB) with emergence traps during the four sampling periods of the present study.

Period	**Total**	**DH**	**CP**	**BM**	**CO**	**WB**
Aug. - Sept. 2012	2,896	2,083	713	27	2	71
Apr. - May 2013	17,848	15,195	1,940	17	1	695
June 2013	6,296	5,983	302	0	6	5
July - Aug. 2013	1,548	1,303	245	0	0	0

4.3 DISCUSSION

In the surroundings of livestock holdings, such as cattle farms, large numbers of suitable hosts are available for *Culicoides* species feeding on vertebrates. According to the present results, suitable breeding substrates are available in this environment for the species of the Obsoletus Group. In this investigation focused on cattle farms, *C. obsoletus* s.s. was the most abundant species and occurred in samples from all investigated habitat types, i.e., dungheaps, cowpats, bark mulch, compost and water-associated substrates. Also according to the literature review (see section 4.1.1), large number of diverse breeding sites are occupied by this species. The review indicated animal dung in form of dungheaps or dung litter, to be among the important breeding sites for *C. obsoletus* s.s.. Correspondingly, the field study demonstrated a clear preference for the development in cattle dungheaps above other habitat types examined. *Culicoides obsoletus* s.s. developed in enormous numbers in this habitat, several hundred individuals per emergence trap were counted on several occasions. Even though the productivity of dungheaps varied strongly between the farms, these data suggest that this habitat type must be considered as the most important source of *C. obsoletus* s.s. in the surroundings of cattle farms. According to the literature review, this species can develop in the dung of various animal species, including sheep dung. Thus, also dung collections on German sheep farms can be considered potential habitats. The species also developed in compost and bark mulch in low density. However, these substrates were only available on a limited number of farms (three farms with bark mulch, three farms with compost).

According to another conclusion of the literature review, *C. obsoletus* s.s. rarely develops in cowpats. This matches the results of the present study, however, with one exception: *C. obsoletus* s.s. occurred in four cowpats at one farm (April - May 2013) in association with unusual large numbers of *C. scoticus*. In no physico-chemical parameters measured, except for a comparably low water content (mean ± standard deviation = 29.7 ± 11.8%) in comparison to the overall water content measured in cowpats (see Table 5.2, 45.1 ± 20%), did these cowpats notably differ from other samples.

The field study examined a large number of water-associated substrates. Although *C. obsoletus* s.s. occasionally emerged from the margins of water bodies according to the literature, such habitats seem to be no important reservoirs for any of the Obsoletus Group species (see section 4.1.1). Possibly, these species avoid substrates that are likely to be inundated. Previously, another researcher postulated that pupae of the subgenus *Avaritia* drown when submerged, and thus, the immature stages do not develop in waterlogged substrates (Nevill et al., 2007). However, one exception was evident in the present study: from the mud around a small pond, interspersed with decaying oak leaves and residues from a near dungheap, considerable numbers of *C. obsoletus* s.s. emerged from this habitat in association with *C. scoticus*. The substrate seemed inconspicuous according to the abiotic character, but the combination of moist substrate with dungheap residues obviously provided suitable conditions for the development of these species.

Aside from the pond and the four cowpats mentioned previously, the present field study identified only a small number of breeding sites of *C. scoticus*. In contrast to *C. obsoletus* s.s., this species rarely occurred in dungheaps. This is in line with the results of Harrup et al. (2013), who found "muck heaps" to be the only breeding site where the immature stages of two species were not found in association. According to the present study and the literature review, a typical habitat for *C. scoticus* is not apparent. *Culicoides scoticus* appears opportunistic in the choice of substrates. Considering that the species is common in light trap catches (e.g., Monteys and Saiz-Ardanaz, 2003; Baldet et al., 2004; Balczun et al., 2009; Nielsen et al., 2010), other, currently unknown, breeding sites probably exist. Further studies should place more emphasis on the investigation of decaying plant material, e.g., leaf litter, as *C. scoticus* repeatedly occurred in habitats of this type according to the literature (see 4.1.1.2).

While cowpats were of limited significance for *C. obsoletus* s.s. and *C. scoticus*, it was clarified that this habitat was by far the most important source of *C. chiopterus* and *C. dewulfi* and these species neither occurred in semi-aquatic substrates, bark mulch or compost, nor were they common in dungheaps. Also the authors of other studies sampled only single specimens were from old composted manure, pine litter, moist straw, soil polluted with chicken manure, fungi and silage residues (Laurence, 1953; Jamnback, 1965; Hackman and Meinander, 1979; González et al., 2013; Zimmer et al., 2013b). Also a recent survey from Belgium reported small numbers of *C. chiopterus* and *C. dewulfi* to occur in various substrates, e.g., in the soil of a livestock trampling area, components of a chicken coop, food residues and molehill soil. However, the majority of individuals emerged from cowpats (Zimmer et al., 2014).

This study pointed out a clear preference of *C. chiopterus* and *C. dewulfi* for cowpats, while for *C. obsoletus* s.s., a strong species-specific preference for dungheaps was evident. This separation gives rise to the question about the decisive factors distinguishing these two habitats. Presumably, the appearance (cowpats = small, dungheaps = large) or the material composition (cowpats = pure dung, dungheaps = dung, urine and straw) play a role for habitat selection. The habitats might also differ with regard to the food supply. The knowledge on food sources of *Culicoides* larvae is limited, but it is assumed that they feed on microorganisms or detritus (Kettle, 1962; Williams and Turner, 1976). This kind of nutrition should be available in both, cowpats and dungheaps. However, a closer look at microbe species communities could reveal potential differences. Moreover, in contrast to cowpats, lying outside on pastures, dungheaps offer warm breeding sites even in winter (see Manuscript VI, section 11.2.1) and are often located close to the stable. Possibly, *C. obsoletus* s.s. prefers to feed on hosts indoors and to lay eggs in the immediate surroundings of the livestock. The species was reported to enter buildings and to emerge from dung inside stables in previous studies (Baldet et al., 2008; Meiswinkel et al., 2008; Zimmer et al., 2010; Ninio et al., 2011b). Against this background, it cannot be excluded that oviposition also takes places inside stables and that the colonised material is later added to the dungheaps outside, from where the midges emerge. In any case, prospective studies need to differentiate between these two types of cattle dung.

CHAPTER 5

IMPACT OF ENVIRONMENTAL FACTORS ON HABITAT COLONISATION BY SPECIES OF THE OBSOLETUS GROUP

This chapter concerns a more detailed analysis of the emergence of *Culicoides* from breeding sites, identified as important habitats of the Obsoletus Group, as described in chapter 4, i.e., dungheaps and cowpats. The emphasis of the appraisal was placed on the relation between environmental parameters characterising these habitats and the distribution and abundance of immature *Culicoides*.

Chapter 5 is based on results published by the journal **Medical and Veterinary Entomology** [→ manuscript III (*Emergence of Culicoides obsoletus group species from farm-associated habitats in Germany*), section 11.1.3].

5.1 INTRODUCTION

Several studies in *Culicoides* research investigated potential relations between the occurrence of juvenile stages and environmental parameters in different breeding sites, e.g., in coastal salt marsh, open grass- and woodland or the littoral of freshwater habitats. Organic matter or mineral contents, substrate pH, but also shade or the presence of certain plants were among the factors suggested to play a role for the distribution of immature *Culicoides* (Jones, 1961; Magnon et al., 1990; Blackwell et al., 1999; Schmidtmann et al., 2000). However, our knowledge on how these characteristics impact the distribution and development of *Culicoides* is still limited (Purse et al., 2015).

The following analysis focuses two clearly defined habitats that were identified as the main breeding sites for three members of the Obsoletus Group in the field study of this thesis (see section 4.2). The number of emerging adult midges provides a measure for the abundance of immature stages in a breeding substrate and the development success of the present larvae and pupae under the prevailing conditions. Therefore, the present chapter analyses the emergence of *C. chiopterus* and *C. dewulfi* from cowpats and *C. obsoletus* s.s. from dungheaps, in relation to potential impacts of several environmental conditions in these habitats, e.g., pH, CN-ratio, substrate moisture or organic matter.

5.2 METHODS: REGRESSION ANALYSIS

The sampling methods of *Culicoides* and the according results, that this analysis is based on, were already described in the previous chapter (see section 4.2.2). Therefore, the method section of chapter 5 focuses on the statistical evaluation of these data, i.e., the analysis of individual numbers sampled with emergence traps on cowpats and dungheaps on the investigated cattle farms (see section 4.2.3) with a multiple regression analysis using the program R (R Core Team, 2014).

R offers several tools for the regression analysis of count data. Thus, different approaches were considered to analyse the relation between *Culicoides* data and the abiotic character of substrates these individuals emerged from, e.g., generalised linear models (overdispersion corrected for with quasi-glm), zero-altered negative binomial models and zero-inflated negative binomial models (function *hurdle* and *zeroinfl*, package "pscl", version 1.4.9, Zeileis et al., 2008). The present data were characterised by excess zeros (absence of Obsoletus Group in 70% of all samples) and overdispersion, caused by both, large count data and the large number of zeros. In particular, zero-altered negative binomial models and zero-inflated negative binomial models are useful methods for handling this kind of issue (Zeileis et al., 2008). However, residual analysis of the results indicated that the models did not explain the present data sufficiently. Therefore, we adopted a two-step approach. In the first step, a linear mixed-effects model (function *lme*, package "nlme", version 3.1 - 120, Pinheiro et al., 2015) was applied on positive counts only, and in the second step, a generalised linear mixed-effects model with binomial distribution (function *glmer*, package "lme4", version 1.1 - 7, Bates et al., 2014) analysed the presence or absence of *Culicoides* in breeding sites. This approach allowed for a log transformation of the count data and implementation of the different sampling campaigns as a random effect in both models to account for seasonal variation within the data.

The results from cowpats and dungheaps were analysed separately, resulting in a total of six models with different response variables, i.e., emergence of the Obsoletus complex from dungheaps: (1a: positive counts, 1b: presence/absence), emergence of *C. chiopterus* from cowpats (2a: positive counts, 2b: presence/absence) and emergence of *C. dewulfi* from cowpats (3a: positive counts, 3b: presence/absence).

Table 5.1 Full set of factors included in the regression analysis of the number of *C. obsoletus* s.s. collected in emergence traps on dungheaps and the number of *C. chiopterus* or *C. dewulfi*, respectively, collected with emergence traps on cowpats. Further parameters of the regression models are summarised in Table 5.3.

	Dungheaps	**Cowpats**
Species	*C. obsoletus* s.s.	*C. chiopterus*/ *C. dewulfi*
Fixed effects	pH phosphor (g * kg^{-1}) CN-ratio organic matter (%) shade (3-level factor) latitude (decimal degree) longitude (decimal degree) moisture (%) trap height (m)	pH phosphor (g * kg^{-1}) CN-ratio organic matter (%) shade (3-level factor) latitude (decimal degree) longitude (decimal degree) water content (%)
Random effect	period (4-level factor)	period (4-level factor)
Models (Table 5.3)	1a - 1b	2a - 3b

Model selection started with the full set of variables given in Table 5.1. Considering potential variations due to the different geographical regions of the farms, the longitude and latitude (decimal degree) of the emergence traps was included as an additional fixed effect to all models. However, the number of sample sites (farms) on the longitudinal and latitudinal gradients was considered too low to allow actual conclusions. Therefore, associated results will not be included in the discussion.

To avoid unequally sized data sets in the modelling process, observations with missing values in explanatory variables were removed prior to the analysis. Furthermore, continuous predictor variables were centred by subtracting their means from the observed scores. In order to minimise the loss of data for the regression analysis, missing values for moisture (due to a technical defect of the moisture probe, 52 values) and water content (cowpats of small size did not always provide enough material for both substrate samples, i.e., chemical analysis and water content, 70 values) were replaced by the mean of observations. The function *corvif* provided by Zuur et al. (2009) calculated generalised variance inflation factors (gVIFs) for the whole data set and for subsets of the different sampling campaigns, in order to avoid multicollinearity among covariates. Values > 3 lead to exclusion of the variable with the highest value and the calculation was repeated until all values were below this threshold. Consequently, the variable potassium was excluded from further analysis.

In order to identify the set of predictors with the highest explanatory power, predictor variables were sequentially dropped from the full model in a backward regression procedure by stepwise excluding the variables with the highest p-value and comparing every new model with the previous model using an ANOVA (Zuur et al., 2009). After dropping one variable, the model was refitted with all remaining variables. These were dropped again sequentially until all remaining variables were significant. The evaluation of the final models depended on the calculating of a conditional coefficient of determination (R^2) (Nakagawa and Schielzeth, 2013; Johnson, 2014) and residual analysis (histograms of Pearson’s residuals, fitted values vs. Pearson’s residuals). The random effect "period" remained in the model if it did not explain any variance (when the standard deviation equalled zero) in order to keep the R^2 values comparable. A confidence level of 5% defined statistically significant differences.

5.3 RESULTS AND DISCUSSION

In the following sections the results of the regression analysis will be discussed separately for each of the included factors. The ranges of these parameters in cowpats and dungheaps are provided in Table 5.2 and the summarised results of the regression analysis in Table 5.3.

The **pH values,** measured in substrates colonised by *Culicoides* in previous studies, range from 3.9 (bog; Williams, 1956) to 9.7 (salt marsh; Lardeux and Ottenwaelder, 1997). In the present study, *C. obsoletus* s.s. successfully developed in dungheap samples with pH values between 5.4 and 9.8 (Figure 5.1, Table 5.2). It was evident that substrate-pH varied considerably within this habitat type and significantly affected the distribution of *C. obsoletus* s.s.: with decreasing pH, the probability for the presence of *C. obsoletus* s.s. and for a higher abundance increased (Table 5.3). In another study, using generalised linear models, the authors reported an association of this species with alkaline to neutral pH levels (Harrup et al., 2013), whereas they measured a notably smaller range of pH in colonised substrates (i.e., 6.6 - 7.6.). However, the trend of a potentially changing abundance with pH might become more obvious in the analysis of data representing a wider pH range, as conducted in the present study.

Substrate pH was the only parameter measured with significant impact on the distribution of all three species, as analysed in the regression analysis. Also the abundance of *C. chiopterus* and the probability for the presence of *C. dewulfi* in cowpats increased with decreasing pH (Table 5.3). According to the literature, the influence of this factor differs within the genus of *Culicoides*: significant positive relationships between immature abundance and pH in bog soil were reported for *C. impunctatus* (measured range: 1.8 - 6.5) while larval densities of *C. nubeculosus* (Meigen) were negatively correlated with pH in mud samples (only mean pH values provided: 7.3 – 8) (Blackwell et al., 1999; Uslu and Dik, 2010). Thus, reliable conclusions considering the impact of the pH value have to be evaluated separately for species and the type of habitat.

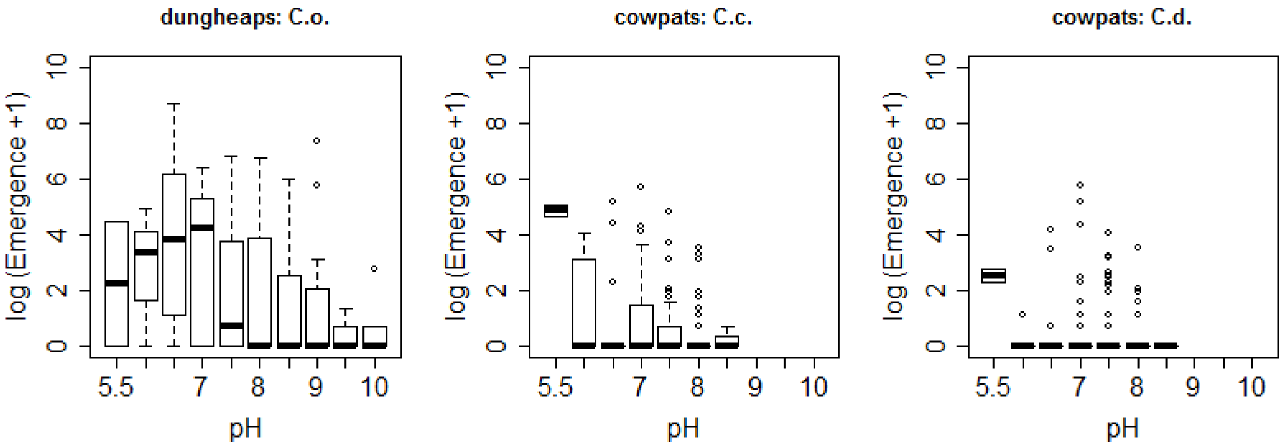

Figure 5.1 Number of individuals of *C. obsoletus* s.s. (C.o.), *C. chiopterus* (C.c.) and *C. dewulfi* (C.d.) emerging from the substrate of dungheaps or cowpats with different pH values (pH rounded to nearest 0.5). The number of emerged individuals is displayed as log(x+1) to allow a better visualisation. The boxplots describe the median (central bar) and the 25% and 75% quartiles (upper and lower boundary of the box, respectively). The whiskers extend to the lowest and highest data points, that are still within 1.5x the interquartile range (= length of the box).

The breeding sites of many *Culicoides* species are described as rich in **organic matter** (e.g., Braverman et al., 1974; Blackwell, 2008; Zimmer et al., 2014). Also animal dung is naturally rich of organic matter – the mean amount measured in the present study was 75 ± 17% (mean ± standard deviation) in dungheaps and 74 ± 19% in cowpats (Table 5.2). It is most likely not a limiting factor in these habitats. This might be related to the fact, that in the present analysis, the percentage of organic matter could not explain the distribution of the dung-breeding species in dungheaps or cowpats. Examinations of other types of developmental sites have partly resulted in different conclusions: according to a study on aquatic habitats, high larval densities of *C. sonorensis* Wirth primarily occurred in breeding sites characterised by "high" amounts of organic matter (Schmidtmann et al., 2000, measured range: 4.3 - 14.4%). The authors discussed that their results probably reflect a preference of this species for habitats contaminated with livestock waste. Instead, *C. gejgelensis* Dzhafarov and *C. festivipennis* Kieffer predominated in sites poor in organic matter (measured range: 2.7 - 11.8%; Uslu and Dik, 2010). Again, the diverging conclusions underline the need to differentiate species-specific preferences in consideration of the character of investigated habitats.

Table 5.2 Ranges of physico-chemical parameters (minimum (min), mean ± standard deviation, maximum (max)) measured in dungheaps and cowpats. The given values either refer to all investigated samples (all samples) or samples that at least one specimen of *C. obsoletus* s.s., *C. chiopterus* or *C. dewulfi* emerged from (n = number of samples examined).

Habitat	Parameter	Observations	n	min	median	mean	max
Dungheap	pH	all samples	219	5.3	7.9	7.9 ± 0.9	9.9
		C. obsoletus s.s.	110	5.4	7.6	7.0 ± 0.9	9.8
	phosphor ($g * kg^{-1}$)	all samples	216	0.1	3.5	3.8 ± 2.2	18.5
		C. obsoletus s.s.	110	0.1	3.3	3.7 ± 2.5	18.5
	C/N	all samples	216	7.6	12.1	13.2 ± 4.0	40.0
		C. obsoletus s.s.	110	8.7	12.1	13.0 ± 4.2	40.0
	moisture (%)	all samples	177	0.0	37.5	34.7 ± 16.0	100
		C. obsoletus s.s.	88	0.0	39.2	35.7 ± 16.8	100
	organic matter (%)	all samples	218	18.4	77.9	74.5 ± 16.9	99.4
		C. obsoletus s.s.	109	24.1	72.4	71.4 ± 17.5	99.4
	trap height (m)	all samples	226	0.0	1.2	1.3 ± 0.9	4.0
		C. obsoletus s.s.	107	0.0	1.0	0.9 ± 0.6	2.5
Cowpat	pH	all samples	221	5.3	7.3	7.2 ± 0.5	8.4
		C. chiopterus	57	5.3	7.1	7.1 ± 0.6	8.2
		C. dewulfi	34	5.3	7.0	6.9 ± 0.6	7.8
	phosphor ($g * kg^{-1}$)	all samples	221	0.2	4.2	4.2 ± 2.4	13.1
		C. chiopterus	57	0.2	4.5	4.3 ± 2.5	10.7
		C. dewulfi	34	0.2	4.2	4.2 ± 2.7	10.7
	C/N	all samples	220	11.1	15.0	15.4 ± 2.5	23.1
		C. chiopterus	57	11.3	15.7	15.9 ± 2.6	23.1
		C. dewulfi	34	12.2	15.5	15.7 ± 2.4	22.1
	water content (%)	all samples	155	6.9	48.3	45.1 ± 20.0	85.8
		C. chiopterus	40	18.7	49.0	48.7 ± 2.6	85.8
		C. dewulfi	24	19.8	41.8	43.4 ± 16.2	76.2
	organic matter (%)	all samples	197	11.9	77.4	74.1 ± 19.4	98.0
		C. chiopterus	50	11.9	74.9	68.7 ± 23.5	97.3
		C. dewulfi	27	16.3	74.8	68.1 ± 26.5	97.3

The regression analysis of this study revealed no effect of the **CN-ratio** on the distribution of *C. obsoletus* s.s. in dungheaps; also the impact on species developing in cowpats appeared to be limited (Figure 5.2, Table 5.3). Overall, the measured CN-ratios were distributed over a more restricted range in cowpats (measured range: 11.1 - 23.1) as compared to dungheaps

(7.6 - 40), reflecting the variability of the latter habitat, e.g., regarding the state of decomposition. The regression analysis indicated that the abundance of *C. dewulfi* increased with the CN-ratio in cowpats (Table 5.2, Figure 5.2) but the reliability of this result is questionable considering the low R^2 of the model (Table 5.3).

Studies investigating the impact of the CN-ratio on the distribution of *Culicoides* in breeding habitats are rare. The examination of dried dung from inside a cowshed, indicated a higher abundance of males of *C. obsoletus* s.s. in substrates characterised by a high CN-ratio (12.5 - 19.5; Zimmer et al., 2010). The authors argued that a ratio between 15 and 30 allows good microbial growth and therefore indicates a good food supply for *Culicoides* larvae. They refer to a study of Williams and Turner (1976), who demonstrated that larvae of the tree-hole species *C. guttipennis* (Coquillett) do not develop in sterilised leaf matter, but only in unsterilised material containing a variety of microorganisms. However, the food sources of larvae of the Obsoletus Group are poorly known and such conclusions therefore require further evidence.

Nevertheless, the CN-ratio is a general measure for the rate of decomposition, and thus, for the microbial growth (Taylor et al., 1989). Provided, the larvae of the Obsoletus Group species feed on microorganisms, this parameter describes the nutritional value of a substrate. The interactions between microorganisms and the decomposition rate are complex and a meaningful interpretation of the CN-ratio ought to require the comprehension of many aspects, e.g., the substrate composition: the enhancement of the decomposition by increasing N is stronger in complex C substrates (i.e., cellulose or plant cell walls) than in labile C substrates (e.g., glucose; Koranda et al., 2014). Compared to cowpats, dungheaps contain higher amounts of complex C-substrates in form of straw. Thus, the same CN-ratio in a dungheap sample and a cowpat might indicate different rates of microbial growth. This argumentation is speculative but could explain the potential impact of the CN-ration on *C. dewulfi* in cowpats, but not for *C. obsoletus* s.s. in dungheaps. However, the regression analysis did not indicate any effect of the CN-ratio on the distribution of *C. chiopterus* in cowpats. According to another study, the increase of N in substrates with high lignin content effectively stimulates decomposition (Knorr et al., 2005). At the same time, results of Zimmer et al. (2013b) indicate a positive impact of a high content of lignin and on the abundance of larvae of the Obsoletus complex in silage residues. Unfortunately, the authors did not measure CN-ratios of the silage. Possibly, a combination of the CN-ratio with other variables, e.g., cellulose or lignin content, could serve as an indirect measure of substrate quality for immature *Culicoides*.

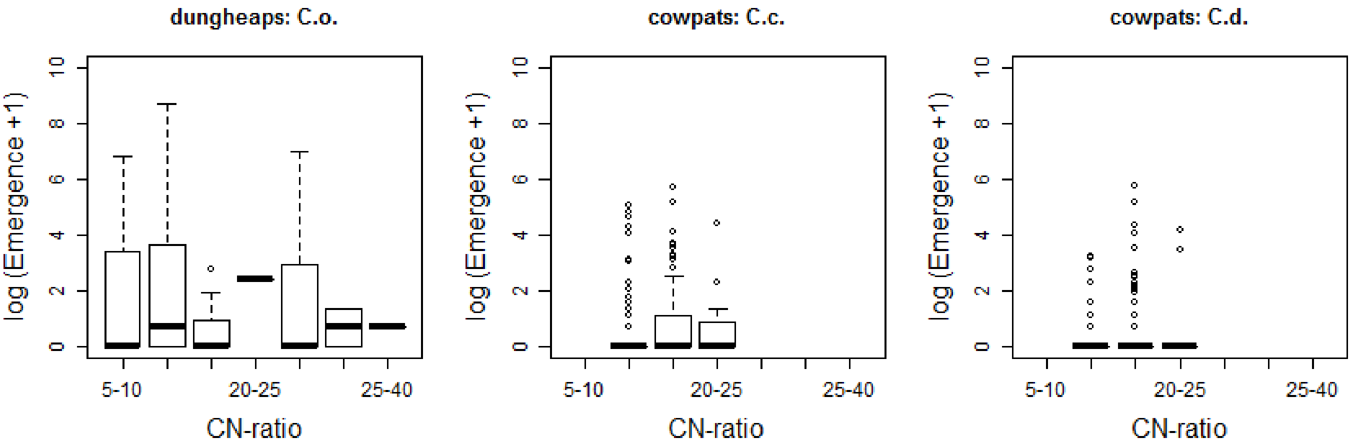

Figure 5.2 Number of individuals of *C. obsoletus* s.s. (C.o.), *C. chiopterus* (C.c.) and *C. dewulfi* (C.d.) emerging from the substrate of dungheaps or cowpats with different CN-ratios. The number of emerged individuals is displayed as log(x+1) to allow a better visualisation (see Figure 5.1 for explanation of the boxplot graphs).

In several studies, investigating the impact of **phosphor** availability in breeding habitats on *Culicoides* distribution, no relation between the phosphor concentration and the emergence of adult or presence of immature *Culicoides* was found (e.g., Battle and Turner, 1972; Schmidtmann et al., 2000; Zimmer et al., 2013a). This also applies to the present study: the concentration of phosphor did not explain the number of emerging individuals – neither in dungheaps nor in cowpats. The species of the Obsoletus Group seemed to tolerate the whole range of measured values (Table 5.2). As an indicator of organic load, high amounts of phosphor are common in agricultural environments (Daniel et al., 1998). Thus, it is not surprising that the species of the Obsoletus Group, that commonly occur habitats associated to livestock (see section 4.1.1) are not notably affected by high phosphor concentrations. Another study revealed a relation between *Culicoides* distribution and phosphor reported diverging results for two species: numbers of *C. nubeculosus* decreased, while the number of *C. festivipennis* increased with the phosphor concentration in different types of mud not associated to livestock (Uslu and Dik, 2010).

The impact of soil **moisture** on the larval development of *Culicoides* has not received much attention in previous research (reviewed by Mellor et al., 2000). In the present study, individual numbers of *C. obsoletus* s.s. increased with substrate moisture in dungheaps, with highest abundances at ~ 40 - 60% (Table 5.2, Figure 5.3). These results are in line with the conclusions of Harrup et al. (2013). Instead, according to the present results, there was no impact of water content on the number of emerging *C. chiopterus* or *C. dewulfi* from cowpats.

Both species successfully developed in cowpats with the water content below 20%. A previous study demonstrated that even the soil moisture underneath cowpats is positively correlated with the number of emerging midges of these species (see Manuscript X, section 11.2.5). However, this does not seem to be true for the actual breeding substrate, i.e., the cowpats.

Possibly, the different results for dungheaps and cowpats might be related to the fact, that if a colonised cowpat dries, immature stages will not have the option to migrate into another cowpat. On the contrast, larvae in a dungheap should be able to move to more favourable positions within the heap, e.g., into substrate regions with a higher moisture. Thus, the development in cowpats possibly requires a higher tolerance towards desiccation. Accordingly, the successful development of *C. chiopterus* and *C. dewulfi* in drying cowpats in the laboratory suggested these species to be quite resistant towards desiccation (see Manuscript VII, section 11.2.2). However, in another study, the vertical movement of *C. brevitarsis* larvae within cowpats was associated with higher substrate moisture (Bishop et al., 1996b). The present study did not allow for an assessment of moisture differences and the position of juvenile stages within cowpats. Thus, it is also possible that *C. chiopterus* and *C. dewulfi* prefer regions with higher moisture within the cowpat, without their development success being notably affected by an overall drying.

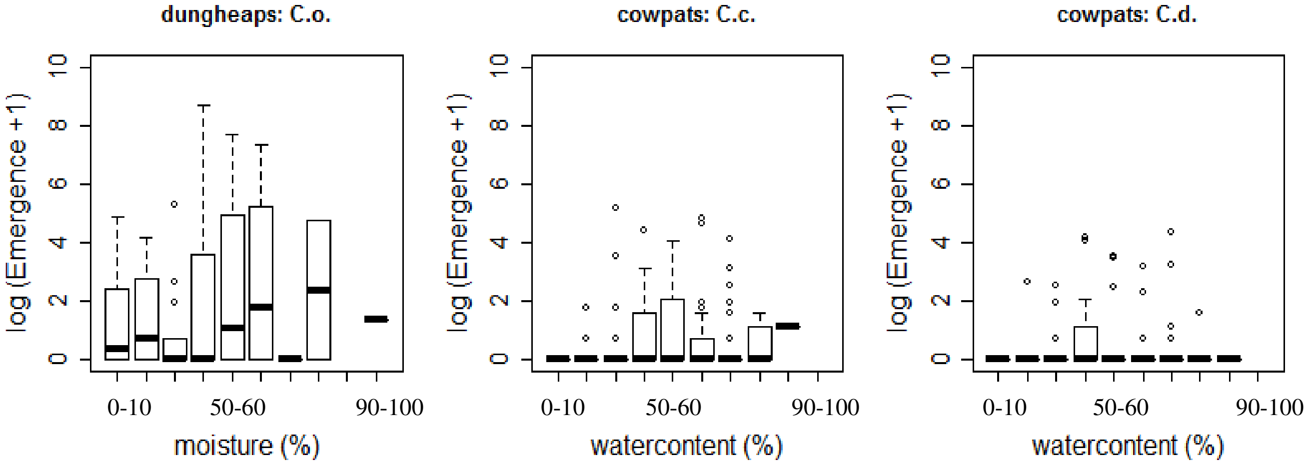

Figure 5.3 Number of individuals of *C. obsoletus* s.s. (C.o.), *C. chiopterus* (C.c.) and *C. dewulfi* (C.d.) emerging from the substrate of dungheaps or cowpats, with different substrate moisture (%), or water content (%), respectively. The number of individuals is displayed as log(x+1) to allow a better visualisation (see Figure 5.1 for explanation of the boxplot graphs).

Besides the chemical characteristics included in the analysis of this study, also a potential impact of **shade** was considered, as temperatures within dungheaps can become very high due to microbial activity. In winter, immature *C. obsoletus* s.s. were extracted from substrates of dungheaps with the temperatures ranging between 7.9 and 38.0 °C (see Manuscript VI, section 11.2.1). Thus, the species generally seems to tolerate high temperatures in the breeding substrate. Temperatures in dungheaps can increase up to 60 °C (Kiel et al., 2009). However, this study revealed no obvious relation between *Culicoides* abundance or presence to the degree of shading (never shaded, temporarily shaded or constantly shaded). Possibly, the reduction of heating by shade is of minor importance for the development of *Culicoides* in temperate climate.

Besides substrate moisture and pH, the individual numbers of *C. obsoletus* s.s. in emergence traps seemed to be notably affected by the position of the traps on the dungheap, i.e., the probability of occurrence decreased with increasing **trap height**. Maximum individual numbers occurred at heights between 0 and 1.5 metres (Figure 5.4) while the trap height ranged from 0 to 4 metres (Table 5.2). A similar preference for lower heights has been indicated by a previous investigation, where the overwintering larvae of the Obsoletus complex were absent from the top layer of a cattle dungheap and most abundant in lower and medium layers (see Manuscript VI, section 11.2.1). Several explanations are possible. The results could indicate unfavourable conditions for immature *Culicoides* in the upper layers of the heaps; possibly related to the age of the dung, as was already suggested in the literature review of breeding sites for *C. obsoletus* s.s (see section 4.1.1.1). Given, fresh dung is regularly added on top of the heaps by the farmers, higher traps refer to fresher dung. Thus, a preference for aged animal litter – possibly presenting an advanced decomposition state – is conceivable. However, no differences of the abiotic character in relation to trap height were evident (not shown). Alternatively, the results might reflect a preference for female oviposition at lower heights. According to Kettle (1962), flight activity of *C. obsoletus* s.s. is more or less confined to the near ground level and also Service (1971) sampled highest numbers of the Obsoletus complex with suction traps at a height of 23 cm, when comparing heights up to 550 cm. While these results refer primarily to unfed (host-seeking) females, trapping methods targeting gravid females, e.g., with ovitraps, are necessary to allow conclusions on the oviposition behaviour.

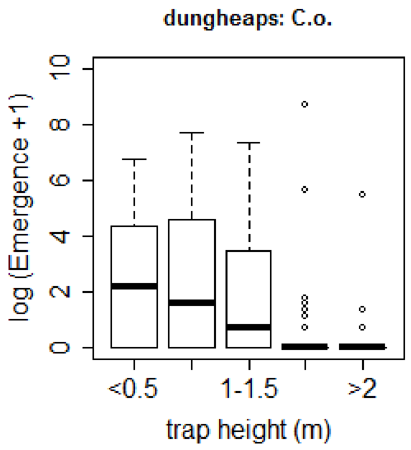

Figure 5.4 Number of *C. obsoletus* s.s. captured with emergence traps on dungheaps at different heights (m). The number of emerged individuals is displayed as log(x+1) to allow for better visualisation (see Figure 5.1 for explanation of the boxplot graphs).

In the present analysis, the **sampling period** was implemented in all models as a random effect, in order to account for potential seasonal variation. The individual numbers differed among sampling periods (see section 4.2.3), but data variation was not explained by this factor by all models (Table 5.3). This result does, of course, not deny a temporal pattern of *Culicoides* emergence as the low number of sampling campaigns in this study merely allows a rough assessment of the seasonal fluctuations of abundance.

Table 5.3 Regression coefficients estimates, standard errors (SE) and *p*-values for fixed effects retained in the positive count models (*lme*) and binomial models (*glmer*, presence vs. absence) and standard deviation (SD) of the random effect "period". The number of observations analysed (n), Akaikes information criterion (AIC) and conditional coefficient of determination (R^2) for final variable sets are given.

Response/ model coefficients	**Estimates**	**SE**	**SD**	***p*-value**	**n**	**AIC**	**R^2**
1a Emergence of *C. obsoletus* s.s. from dungheaps: positive counts					105	446.3	0.27
pH	-0.94	± 0.21		< 0.001			
Moisture	0.04	± 0.01		< 0.01			
Period (random effect)			0.51				
1b Emergence of *C. obsoletus* s.s. from dungheaps: presence vs. absence					210	239.8	0.43
pH	-0.69	± 0.21		< 0.001			
Trap height	-1.36	± 0.22		< 0.001			
Longitude	-0.26	± 0.1		< 0.01			
Latitude	-0.22	± 0.09		< 0.05			
Period (random effect)			0				
2a Emergence of *C. chiopterus* from cowpats: positive counts					50	171.9	0.34
pH	-1.36	± 0.30		< 0.001			
Period (random effect)			0.0004				
2b Emergence of *C. chiopterus* from cowpats: presence vs. absence					192	210.6	0.14
Longitude	-0.36	± 0.10		< 0.001			
Period (random effect)			0.20				
3a Emergence of *C. dewulfi* from cowpats: positive counts					27	102.6	0.22
CN-ratio	0.29	± 0.10		< 0.05			
Period (random effect)			0.0004				
3b Emergence of *C. dewulfi* from cowpats: presence vs. absence					193	149.9	0.18
pH	-0.88	± 0.40		< 0.01			
Longitude	-0.34	± 0.13		< 0.05			
Period (random effect)			0				

5.4 CONCLUSIONS

According to the present data, immature stages of *C. obsoletus* s.s., *C. chiopterus* and *C. dewulfi* tolerate wide ranges of several physico-chemical parameters. This particularly applies to pH, phosphor, CN-ratio and the percentage of organic matter. Animal dung is naturally characterised by a high organic load, and thus, parameters as organic matter or phosphor concentrations might not be limiting the presence of dung-breeding species. Instead, substrate pH helped to explain the spatial distribution in dungheaps and cowpats alike. Moisture positively affected the abundance of *C. obsoletus* s.s. in dungheaps, whereas the water content in cowpats did not impact the abundance of *C. chiopterus* and *C. dewulfi*. These species were assumed to be more resistant towards desiccation of their breeding sites. Further studies should place particular emphasis on the assessment of substrate temperature, the activity of microorganisms, or the combination of the CN-ratio with lignin or cellulose contents in order to explain the occurrence of immature *Culicoides* in more detail.

CHAPTER 6

IMPACT OF FREEZING ON THE EMERGENCE OF *C. CHIOPTERUS* AND *C. DEWULFI* FROM BOVINE DUNG

Chapter 6 presents an experimental study on the two species of the Obsoletus Group that primarily develop in cowpats – *C. chiopterus* and *C. dewulfi*. The development success of overwintering larvae was investigated after artificial freezing of the breeding substrate. Additionally, a monitoring of the temperatures within cowpats on a pasture helped to assess the temperature range overwintering larvae are naturally confronted with during winter.

This chapter is based on a manuscript published by the journal **Veterinary Parasitology** [→ manuscript IV (*Impact of freezing on the emergence of Culicoides chiopterus and Culicoides dewulfi from bovine dung*), section 11.1.4].

6.1 INTRODUCTION

In 2006, northern Europe was heavily affected by the BTV epidemic (see section 1.3). After a putative vector-free period during the following winter, the bluetongue disease re-emerged in 2007. It is still not completely clear, how BTV could persist during the winter of 2006 (Wilson et al., 2008). Since a mild winter followed the devastating outbreak of BTV in 2006, the question appeared whether a more severe winter would have reduced the emerging *Culicoides* population in spring, resulting in a lower transmission risk. Therefore, this study aimed at a better knowledge on overwintering mechanisms of the vector species and the susceptibility towards sub-zero temperatures.

Many *Culicoides* species in temperate regions overwinter in the larval stage (Becker, 1960; Jones, 1967; Szadziewski et al., 1997). Presumably, this also applies to the species of the Obsoletus Group as high numbers of *C. chiopterus* and *C. dewulfi* were extracted from cowpats in winter and the evaluation of head capsule measurements identified these individuals as fourth instar larvae (see section 3.3.1, Table 3.3). Moreover, both species abundantly emerged from cowpats in spring (see section 4.2.3). Also *C. obsoletus* s.s. emerged in large numbers from dungheaps during this sampling period and larvae of this species were extracted in winter from this habitat (see Manuscript VI, section 11.2.1). Due to microbial activity, the temperature in dungheaps can remain quite high also at ambient temperatures below zero. This provides adequate conditions for the survival of immature stages at hostile temperatures (see Manuscript VI, section 11.2.1). However, it is most questionable if this also applies to cowpats, considering the comparably small organic mass. Therefore, the present study investigates the survival of overwintering *C. chiopterus* and *C. dewulfi* at sub-zero temperatures, to assess the potential impact of severe winters on the population.

In order to assess the temperature range that immature *Culicoides* in this habitat are naturally exposed to, the temperature was monitored in cowpats on a pasture during winter. Moreover, overwintering larvae within cowpats were frozen in the laboratory to investigate subsequent emergence. According to a preliminary study, *C. chiopterus* survives freezing at -15 °C (Künkele, 2013). Therefore, we investigated the emergence after freezing at -18 °C and -21 °C to outline the temperature level precluding survival.

6.2 METHODS

The monitoring of the temperature within cowpats started two month prior to the main experiment. The pasture selected for the study belonged to an organic cattle farm near Oldenburg, already described in chapter 3 (see section 3.2.1). Three round cowpats of similar diameter and height were chosen (about 30 and 4 cm, respectively). The temperature was measured hourly with miniature dataloggers (ø = 1cm; iButton DS1921G, Maxim Integrated, San Jose, CA, USA) inserted into the top half and the bottom half of each pat. Air temperature was simultaneously measured at the edge of the pasture (10 to 20 metres away from the cowpats), at a height of one metre (Hobo Pro v2, ONSET, Bourne, MA, USA). Several days prior to the main study, small subsamples (approximately 10 g each) were collected from the edges of 15 cowpats. *Culicoides* larvae were extracted from the samples via Berlese (see section 3.2.3) to ensure that only colonised cowpats were used in the experiment. In 12 cowpats, *Culicoides* larvae were found and ten of these "positive" cowpats were randomly selected for further use. These cowpats were cut out as a whole together with the soil layer (down to 3 - 4 cm) underneath and transferred to the laboratory in January 2014.

Grazing had been stopped in the foregoing autumn. Thus, cowpats used for the experiments were of the minimum age of three to four months. Each pat was divided into three 120° pieces of approximately equal size. One subsample of each cowpat was frozen for 48 hours at -18 °C and another one at -21 °C in separate deep freezers. The third subsample, stored outdoors, served as a control. During freezing, miniature data loggers (ø = 1cm; iButton DS1921G, Maxim Integrated, San Jose, CA, USA), carefully inserted into the centre of the subsamples, monitored the temperature within the cowpats. Additionally, the temperature in the deep freezers was measured (HOBO Pendant® Temperature/Alarm Data Logger 8K, ONSET, Bourne, MA, USA). The freezing treatments started approximately one hour after the sampling. After the exposure, all samples were placed in quadratic emergence traps, described in chapter 3 (see section 3.2.2).

The emergence traps maintained in a greenhouse at natural daylight conditions and a rearing temperature of 16.0 ± 2.9 °C (Hobo Pro v2, ONSET, Bourne, MA, USA). A solution of 20% NaCl saline, supplemented with a drop of detergent, served for the preservation of emerging insects flying in the collection containers. Cowpat samples were moistened with tap water and trapped insects were removed every second or third day. Female *Culicoides* midges were sorted out after emergence and identified to species level according to chapter 2 (see section 2.4). Males were identified using the key of Campbell and Pelham-Clinton (1960).

Numbers of emerged individuals from different treatments were compared using the Wilcoxon signed-rank test using a confidence level of 5% to define statistically significant differences (R Core Team, 2011).

6.3 RESULTS

A total of 1,361 *Culicoides* midges emerged from the control samples, with a high variation of individual numbers among the cowpats (62.1 ± 55.5 per subsample, Table 6.1). Of these midges, 621 (45.6%) were *C. chiopterus* (290 males, 331 females) and 711 (52.2%) *C. dewulfi* (563 males, 148 females). From samples frozen at -21 °C no *Culicoides* emerged, while a total of 33 *C. chiopterus* (12 males, 20 females) successfully developed and emerged from three out of 10 subsamples, previously frozen at -18 °C. The minimal temperature within these subsamples, -18.2 ± 0.9 °C, was reached towards the end of the 48 hours (46.3 ± 1.9 hours). Subsamples frozen at -21 °C reached a minimal temperature of -23.4 ± 2.4 °C after 42.7 ± 4.8 hours. In comparison with the control samples, significantly fewer *C. chiopterus* individuals emerged from subsamples frozen at -18 °C (Wilcoxon signed-rank test, $n = 10$, $p < 0.001$).

According to the temperature monitoring on the pasture, it is unlikely that the overwintering larvae were confronted with freezing in advance to the main experiment. The average temperature measured within cowpats between 20.11.13 and 23.02.14 was 3.5 ± 2.3 °C. When air temperature sank below 0 °C, cowpat temperatures remained close to 0 °C (Figure 6.1).

Table 6.1 Emergence of *C. chiopterus* (*C.ch.*) and *C. dewulfi* (*C.d.*) from subsamples (C1 - C15) of cowpats maintained at -18 °C for 48 hours and non-frozen samples (control) and minimal temperature (°C (min) reached in frozen subsamples. No *Culicoides* emerged from subsamples maintained at -21 °C (n.a. = data not available due to logger defect). The mean temperature is given with the standard deviation (SD).

Sample	**-18 °C**		**-21° C**		**Control**	
	C.ch.	°C (min)	*C.*	°C (min)	*C.ch.*	*C.d.*
C1	0	-19.4	0	-26.2	81	38
C2	29	-17.1	0	-20.9	66	37
C3	0	-19.0	0	-22.0	182	177
C5	0	-17.0	0	-22.4	53	171
C6	0	-17.1	0	-21.1	17	9
C8	1	-17.6	0	-20.6	41	52
C9	3	-18.4	0	-25.8	22	45
C12	0	-18.0	0	-25.8	141	141
C13	0	-19.1	0	-25.8	10	35
C15	0	-19.0	0	n.a.	8	6
Sum/ mean ± SD (°C)	33	-18.2 ± 0.9	0	-23.4 ± 2.4	621	711

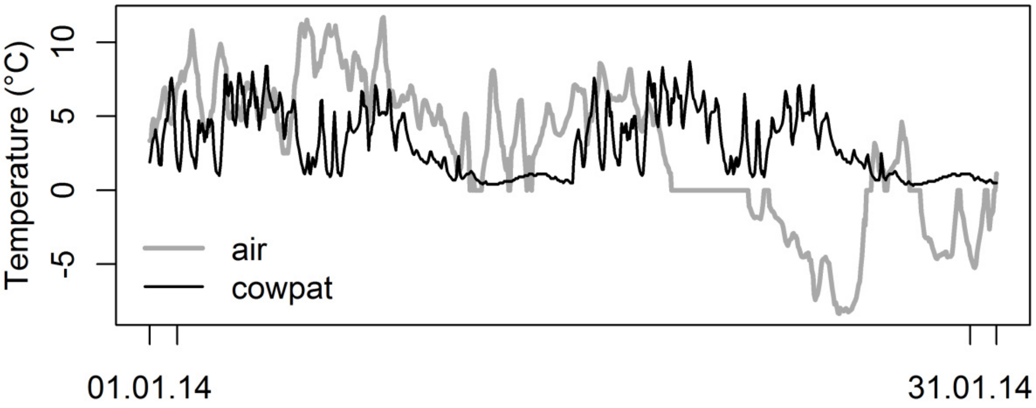

Figure 6.1 Hourly measured air temperature (◦C, grey line) on a pasture in north-western Germany (measured at a height of one metre) in comparison to the average temperature measured within cowpats on the pasture (black line) from 1st to 31st January 2014 (temperature was measured within three cowpats, in the top half and the bottom half).

6.4 DISCUSSION

Larvae of *C. variipennis*, the primary vector of BTV in North America (reviewed by Tabachnick, 1996), are able to survive freezing at a temperature down to -2.2 °C over a period of six weeks (62% of larvae exposed to this temperature; Rowley, 1967). This species also survived icebound in frozen pond mud (Vaughan and Turner, 1987). The present study demonstrates the competence of larvae of another veterinary important species, *C. chiopterus*, to survive temperatures far below 0 °C. The lowest temperature in subsamples, from which *Culicoides* emerged, was -18 °C, while no specimens emerged after freezing at -21 °C. The lower threshold for survival of *C. dewulfi* is assumed to be a higher temperature, as this species was abundant in the control samples, but did not emerge from any frozen samples. This indicates a lower resistance towards the freezing of the breeding substrate. *Culicoides* are generally capable to survive in harsh climates and, in contrast to *C. dewulfi*, *C. chiopterus* also occurs in various regions in Russia, including Western Siberia (Sprygin et al., 2014).

Being ectotherm and, thus, not able to regulate body temperature, only few insect species are capable to survive the actual freezing of the body liquids. However, there are many examples of taxa that can decrease the temperature point, at which their body fluids freeze, below zero: based on the regulation with polyols, the intracellular – and in some taxa also the extracellular fluids – remain liquid (Lee et al., 1993; Dettner and Peters, 2011). It is unknown what mechanisms allow *C. chiopterus* and *C. dewulfi* to survive exposure to sub-zero temperatures. Glycerol levels are elevated in winter larvae of *C. variipennis* but not in individuals sampled during summer (Vaughan and Turner, 1987) and glycerol is among the most common cryoprotectants in overwintering mechanisms of insects (; reviewed by Lee et al., 1993).

The monitoring of winter temperatures in cowpats revealed that *C. chiopterus* and *C. dewulfi* were not confronted with freezing of the breeding substrate during the mild German winter 2013/2014. The temperature of the upper soil layer does not directly follow air temperature below 0 °C, but remains near the freezing point until the ground surface is completely frozen (Beltrami, 2001). Accordingly, the temperature within cowpats should remain close to the freezing point until all water within the dung has frozen, which seems to require lower temperatures than those observed during the present monitoring. Sub-zero temperatures in topsoil occur in Germany but temperatures down to -18 °C are not common (National Meteorological Service of Germany, 2014a, 2014b). According to these data and the capability of a part of the immature population of *C. chiopterus* to survive freezing of the breeding substrate down to -18 °C, a significant reduction of the overwintering population of this species by freezing does not seem probable. Further research will be necessary to determine the exact temperature point and duration of freezing that will have a notable impact on the *Culicoides* population, also with regard to *C. dewulfi*, as the species appears less tolerant towards freezing. More detailed information will allow a better assessment of the potential survival of overwintering larvae during severe winters.

CHAPTER 7

IMPACT OF FLOODING ON LARVAE AND PUPAE OF *C. CHIOPTERUS* AND *C. DEWULFI*

Chapter 7 presents a detailed experimental study investigating the impact of flood events of different duration on the emergence of immature stages of *C. chiopterus* and *C. dewulfi*. The behaviour of larvae in water and the development success after flooding larvae and pupae was investigated. The results are discussed in light of the potential adaption to the development in terrestrial habitats.

The publication, this chapter is based on, is currently under review by the journal **Veterinary Parasitology** [→ manuscript V (*Experimental studies on the impact of flooding on larvae and pupae of dung-breeding Culicoides (Diptera: Ceratopogonidae) with notes on the behaviour of immature stages*), section 11.1.4].

7.1 INTRODUCTION

In the different habitats of immature *Culicoides*, e.g., streams, salt-marsh, tree-holes, fungi or animal dung (Kettle and Lawson, 1952; Murray, 1957; Buxton, 1960; Schmidtmann; 2006) the hydrological conditions differ strongly. Some species prefer to develop in waterlogged habitats, as *C. nubeculosus* and *C. circumscriptus* Kieffer (Downes, 1950; Foxi and Delrio, 2010). Other species seem to avoid inundated substrates, as those of the subgenus *Avaritia* (Nevill et al., 2007). *C. imicola*, for example, is common in moist and muddy habitats, usually lacking surface-water (Braverman and Galun, 1974; Braverman, 1978; Mellor and Pitzolis, 1979; Foxi and Delrio, 2010). *C. chiopterus* and *C. dewulfi* develop in cowpats, which are among the driest breeding habitats colonised by *Culicoides* (Kettle and Lawson, 1952).

Many species possess behavioural or physiologic adaptations for environmental changes in the respective habitat, e.g., for flooding. While the larvae of *C. nubeculosus*, *C. circumscriptus* or *C. variipennis* are good swimmers (Megahed, 1956; Becker, 1960; Linley, 1986), *C. brevitarsis* or *C. imicola* lack this ability (Cannon and Rye, 1966; Bellis et al., 2014). Pupae of *Culicoides* are classified into four groups according to the behaviour in water (Dyce and Murray, 1966; Nevill, 1967). In group 1, pupae can float to the surface but are unable to submerge. The species of group 2 are capable of both, i.e., floating and sinking back under water. Pupae of the third group are unable to float and will burrow into the substrate when flooded. In the fourth group, pupae of *C. imicola* do not float but remain on the ground.
Larvae of the salt-marsh species *C. furens* (Poey) survive immersion for up to 14 days (Magnon et al., 1990). However, in terrestrial species, the resistance to flooding has rarely been investigated. In the dung-breeding species *C. chiopterus* and *C. dewulfi* flooding of the breeding habitat prevents an emergence of adults (see manuscript VII, section 11.2.2) but it remained unclear if the results were related to a sensitivity of larvae or pupae. A different susceptibility of both stages is known for other species of Diptera and other abiotic factors, e.g., a higher or lower resistance of pupae than larvae to freezing (e.g., Mugnano et al., 1996; Jensen et al., 2007). Therefore, the present study investigates the behaviour of larvae in water and the impact of short and long term flooding on the larvae and pupae of these two species separately.

7.2 METHODS

7.2.1 SAMPLING OF *CULICOIDES* LARVAE

Culicoides larvae originated from cowpats, sampled on the pasture described in section section 3.2.1. Larvae were extracted using the Berlese method (see section 3.2.3), collected in tap water and morphologically identified to species level according to the key of Kettle and Lawson (1952). In September and October 2015, larvae were sampled from 20 different cowpats for the recording of the motion sequences of larvae in water. The sampling for the flooding experiments (20 cowpats) took place in February and March 2015. In order to determine the pupation rate in water, further larvae originated from a total of 45 cowpats in July and August 2015.

7.2.2 RECORDING THE MOTION SEQUENCES OF LARVAE IN WATER

After the transfer of larvae into water, these were filmed for at least 15 minutes per specimen, using a webcam (Microsoft LifeCam VX-3000), in combination with a stereomicroscope at low magnification (10x). The motions of 21 larvae at room temperature were analysed and the rate of two different types of larval body motions determined, head-to-tail flexions and sinuous flexions (Linley 1986; Cannon and Rye, 1966). In the head-to-tail flexion, the larval body strongly bent, head and tail approaching each other and sometimes touching; then head and tail slowly weaved to the other side until they were close again. The head-to-tail flexions were rarely performed regularly, and the rate was calculated on the basis of several single flexions, i.e., a single weave at one side from the head to the tail. The second type, the sinuous flexions, resembled a snake like motion. A complete flexion was defined as the weave to one side and back, returning the head to the initial position (see figures and detailed description of swimming *C. variipennis* provided by Linley (1986)). This definition was adopted for the present study, and the calculation of sinuous flexion rate based on the number of complete flexions per second.

After the records, larvae were reared to adults in order to determine the gender and verify the identification of larvae by identifying the adult females following the key in chapter 2 (see section 2.4) and the males using the key of Campbell and Pelham-Clinton (1960). Therefore, larvae maintained in glass petri dishes (60x15mm), filled to one third with moistened sterile silica sand (Rosnerski, Königslutter, Germany) on a bottom layer of paper tissue. In a preliminary study, the sand presented a sufficient maintenance substrate for fourth instar larvae of *C. chiopterus* and *C. dewulfi*.

7.2.3 FLOODING AND MAINTENANCE OF LARVAE

The dishes described in the previous section were also used for the flooding experiments, because the sand does not disperse if the dishes are flooded and facilitates the detection of immature stages and emerged adults. Groups of ten larvae per petri dish were transferred. After 24 hours (time for larvae to dig into the substrate) a total of 100 larvae of *C. chiopterus* and 100 larvae of *C. dewulfi* were flooded for 24 hours and the identical number for ten days. Ten other groups, the controls, were not flooded. For flooding, tap water was carefully added into the petri dishes up to two thirds of the dish. After the period of exposure, the water was carefully withdrawn with a pipette under visual control using a stereomicroscope. The dishes maintained at room temperature (20.4 ± 1.1 °C) and natural daylight conditions. After emergence of adult *Culicoides*, the dishes were opened within a small gauze bag until all adults had left the dish, attracted by the light of a window nearby. This procedure continued until no further adults emerged for at least 14 subsequent days. Generally, no pupae and larvae were detected afterwards. Species determination of the collected adults verified larval identifications (see above). The effect of the different treatments was statistically evaluated by comparing the number of adults in control and flooded groups, using the two-sample proportions test of the programme R, version 3.1.2 (R Core Team, 2014).

7.2.4 PUPATION OF LARVAE IN WATER

After flooding of larvae for ten days exposures, many pupated in water. Since these larvae originated from cowpats collected during winter, a further experiment aimed to clarify, if summer larvae will also pupate when flooded for a longer period of time, and whether they are able to interrupt the development under such unfavourable conditions. For this purpose, 45 larvae of *C. chiopterus* and 68 larvae of *C. dewulfi* were transferred as small groups (2 - 20 larvae) into petri dishes filled with tap water (without sand) and kept at room temperature

(23.4 ± 1.4 °C) and natural daylight conditions. After pupation in water, the direct transfer to new dished containing tap water followed (1 pupa per dish). Ten days after the transfer of the larvae into water, all pupae (Figure 7.1) and larvae were assigned to single dishes containing sand. Emerged adults were removed daily to determine the gender and species of adults (see above). Proportion tests compared the development success of individuals that pupated in water to the number of individuals that did not show this behaviour. The same test compared the number of pupated individuals in water of summer and winter populations.

Figure 7.1 Pupae of *C. dewulfi* laying on the substrate (A) and embedded in the substrate with the cephalothorax protruding (B).

7.2.5 FLOODING AND MAINTENANCE OF PUPAE

Larvae of *C. chiopterus* and *C. dewulfi* maintained in the petri dishes and were controlled daily for pupations. After recording the location of pupae (lying on the sand or under the paper tissue or embedded into the sand), they were carefully transferred within the surrounding substrate into the substrate of new dishes, using a micro-spatula. Pupae that lay loosely on the sand were transferred in the same position. If the cuticula of a fresh pupa had not hardened, the transfer took place on the next day. Directly after the transfer, ten groups, each composing of ten pupae of each species, flooding for periods of 24 hours or ten days followed. Transfer of the control groups, 50 pupae of each species, took place without flooding. After removal of the water from the flooded groups, all dishes were controlled daily for emerged adults, in order to remove them for the determination of species and gender (see above).

7.3 RESULTS

7.3.1 MOTION SEQUENCES OF LARVAE IN WATER

The larvae used in these experiments developed to six males and eight females of *C. chiopterus* and one male and one female of *C. dewulfi*. Another five females belonged to the Obsoletus complex. In no species did head-to-tail flexions result in swimming forward or backward. These flexions (single weaves) occurred at a rate of 0.8 ± 0.2 flexions/second (f/s, n = 14) in *C. chiopterus* and 0.7 ± 0.1 f/s (n = 2) in *C. dewulfi* larvae. The sinuous flexions did, at the most, cause minimal forward locomotion in water. In *C. chiopterus* a rate of 1.6 ± 0.4 f/s (n = 7) and in *C. dewulfi*, a rate of 1.3 f/s (n = 1) was determined. The larvae of the Obsoletus complex moved twice as fast (3.0 ± 0.4 f/s, n = 5). Beside these defined motions, the *Culicoides* larvae often showed other irregular or interrupted movements or remained motionless for up to several minutes.

7.3.2 BEHAVIOUR AND SURVIVAL OF FLOODED LARVAE

After the transfer of larvae into the dishes, some burrowed immediately into the substrate. However, most individuals crawled around on the substrate for several hours. Within 24 hours, most larvae had burrowed into the substrate. A total of 93 *C. chiopterus* developed to adults in controls, 91 after flooding for 24 hours and 50 individuals after flooding for 10 days, in the latter group significantly less compared to controls ($p < 0.001$) (Table 7.1). Less adults of *C. dewulfi* developed successfully under the same conditions, i.e., 78 out of 100 control larvae. In the group of larvae flooded for 24 hours, 87 adults emerged, but significantly less, four, after flooding for a period of ten days ($p < 0.001$ in two-sample proportion test). Among the larvae, flooded for ten days, 48 *C. chiopterus* and 84 *C. dewulfi* pupated in water. All species identifications of emerged adults confirmed the identification of larvae.

Table 1 Development success of larvae and pupae of *C. chiopterus* and *C. dewulfi* after flooding for 24 hours or 10 days.

		Larvae			**Pupae**		
Species	Treatment	n	Emergence rate [%]	ratio ♂:♀	n	Emergence rate [%]	ratio ♂:♀
C. chiopterus	Control	100	93	1.0	50	48	1.0
	flooding: 24 hours	100	91	0.7	100	77	1.2
	flooding: 10 days	100	50	0.7	100	0	
C. dewulfi	Control	100	78	0.7	50	45	2.6
	flooding: 24 hours	100	87	0.9	100	90	1.0
	flooding: 10 days	100	4	0.3	100	0	

7.3.3 PUPATION OF LARVAE IN WATER

Of the larvae obtained from cowpats during summer and maintained in tap water for ten days, 26 *C. chiopterus* (57.8%) and 41 *C. dewulfi* (60.3%) pupated in water. Of the specimens that had pupated in water, four *C. chiopterus* (15.4%) and one *C. dewulfi* (2.4%) successfully finished the development after removal from the water. Another three *C. dewulfi* died during the process of eclosion. In the groups of larvae that did not pupate in water, significantly more developed to adult midges in comparison to those that did pupate in water, i.e., eight out of 19 *C. chiopterus* (42.1%) and six out of 27 *C. dewulfi* (22.2%, $p < 0.05$ in two-sample proportion test). Of larvae extracted in summer, significantly less *C. dewulfi* pupated in water when compared to larvae extracted in winter ($p < 0.01$), whereas no significant difference was evident in *C. chiopterus*.

7.3.4 BEHAVIOUR AND SURVIVAL OF FLOODED PUPAE

Some pupae lied on the sand (23.3% *C. chiopterus*, 23.8% *C. dewulfi*) or under the paper tissue at the bottom of the dish (19.3% *C. chiopterus*, 13.2% *C. dewulfi*). However, the majority (56.4% *C. chiopterus*, 60.3% *C. dewulfi*) pupated in narrow burrows in the sand with the head or cephalothorax protruding. Sometimes, larvae with dilated thoracic segments were observed sitting in such burrows.

No pupae floated during flooding. Burrowed pupae digged themselves out the substrate and lay on the sand, moving only occasionally. Ninety-six and 90% out of 50 control pupae of *C. chiopterus* and *C. dewulfi*, respectively, developed to adults (Table 7.1). Out of the 100 pupae flooded for 24 hours, 77 adults of *C. chiopterus* and 90 of *C. dewulfi* emerged, in the latter statistically significantly less than in controls ($p < 0.001$ in two-sample proportion test). No pupae survived flooding for a period of ten days. In the control groups (transfer to new petri dish without flooding, 50 individuals of each species) nearly all individuals successfully developed to adult *Culicoides*, i.e., 48 *C. chiopterus* and 45 *C. dewulfi* and (Table 1).

7.4 DISCUSSION

A good understanding of the interaction of *Culicoides* with the environment is important in light of the veterinary importance of several species of this genus. A better knowledge of the biology of immature stages, forming the basis of any adult population with the ability to transmit pathogens, could be particularly helpful, e.g., for the development of control measures. However, our knowledge on the ecology of the immature stages of *Culicoides* is still very poor. The present study aimed to provide information on the behaviour and reactions towards changes of the environmental conditions, by flooding the larvae and pupae.

The larvae of almost all *Culicoides* species are competent swimmers and show typical rapid swimming flexion when placed into water (Linley, 1986). The rates of these movements of *Culicoides* larvae vary. In some species they are "so rapid that a larva will appear only as a blur" (Williams, 1951, p. 174). *C. chiopterus* and *C. dewulfi* were not able to swim, but showed sinuous flexions in water, as those described by Linley (1986). However, the flexion rates proved lower than that of swimming *C. variipennis* (10.8 ± 0.2 f/s) and did hardly result in forward locomotion in water. Slow flexions serve *Culicoides* larvae for the movement in wet particulate or fibrous substrates (Linley, 1986) and thus, might be of benefit in mediums of higher viscosity, e.g., the more liquid regions of a cowpat. The "slow head to tail flexion at a rate of less than once per second" described for *C. brevitarsis*, an Australian species developing in cowpats (Cannon and Rye, 1966, p. 8), was also observed in both species in the present study. Cannon and Rye (1966) suggested this behaviour, along with the inability of *C. brevitarsis* to swim or float, to be an adaption to the development in terrestrial habitats. In the present study, the rate of sinuous flexions in larvae of the Obsoletus complex was twice as fast as in *C. chiopterus* and *C. dewulfi*, possibly indicating an adaption to other kinds of substrates. *C. obsoletus* s.s. is able to develop in a wide range of habitats, e.g., in leaf litter, marshland, tree holes, river edges and dungheaps (Kettle and Lawson, 1952; Murray, 1957; González et al., 2013; Harrup et al., 2013).

Because larvae of some species of *Culicoides* cannot escape from flooded habitats by swimming, such changes of the habitat can affect the survival. The larvae of *C. imicola* survived immersion for at least 13 days (Nevill, 1976). *C. chiopterus* and *C. dewulfi* in cowpats do not develop to adults after permanent flooding of this habitat for 31 days or alternate flooding for every third day (see manuscript VII, section 11.2.2). In the present study, flooding for 24 hours had little or no effect on the development success of larvae while flooding for ten days negatively affected the larvae of both species tested, with considerably less larvae of *C. dewulfi* surviving this exposure.

Pupation in water is not common in *Culicoides.* Kettle (1962) concluded that pupation does not occur when larvae are flooded. The mature larvae of some *Culicoides* species migrate into drier habitat regions in order to pupate (Borkent, 2015). According to Bidlingmeyer (1957), *C. furens* pupates only above the waterline, and too much water also hampers the pupation of *C. nubeculosus* (Megahed, 1956). Instead, larvae of *C. imicola* species pupated in water but they died soon afterwards (Nevill, 1967). In the present study, also submerged larvae of *C. dewulfi* and *C. chiopterus* pupated. This behaviour was not restricted to overwintering individuals, but also evident in summer larvae. Both species continued their development under unfavourable conditions, i.e., when being flooded, even though it decreased the probability of survival. Therefore, it seems to be no response to the conditions, but a continuation of development after the initiation of the pupation process.

Pupae seem to be less resistant to flooding than larvae. The pupae of *C. imicola* survived about 7 days less than larvae and died within one day after removal from the water (Nevill, 1976). This different resistance was also evident in the present study. The flooding for 24 hours did not affect larvae, but significantly reduced the developmental success of pupae of *C. chiopterus*. In the field, this situation occurs when pastures are temporarily flooded after heavy rains, but drain soon afterwards. Flooding of the pupae for ten days completely prevented successful development in both species. The different sensitivity of both stages could be related to the oxygen supply. *Culicoides* larvae are apneustic and rely on cutaneous respiration. Instead, pupae (of aquatic species) float to the water surface to obtain oxygen with prothoracic respiratory horns (Kettle and Lawson, 1952; Kettle, 1977; Nevill et al., 2007).

Thus, pupae, that are not able to float, might suffocate more rapidly than the larvae of the same species. The pupae of many species are able to float when flooded (Dyce and Murray, 1965), but *C. imicola*, *C. brevitarsis* and also the species of the Obsoletus Group lack this ability (Jamnback, 1965; Cannon and Rye, 1966; Nevill, 1967). This appears to be characteristic f or the species of the subgenus *Avaritia* that usually develop in moist but not waterlogged substrates (Nevill et al., 2007). The fact, that the larvae of *C. dewulfi* and *C. chiopterus* are not able to swim and the pupae not able to float, is presumably related to the development in cowpats, a habitat, in that the immatures should be rarely confronted with long lasting inundations.

C. dewulfi is less resistant towards temperatures below 0 °C than *C. chiopterus* (see chapter 6). The species was also more susceptible towards flooding. Possibly, *C. dewulfi* requires more constant conditions in the breeding habitat. Moreover, *C. dewulfi* and *C. chiopterus* develop in close coexistence in a spatially confined habitat. Since two species in the same biocenosis cannot occupy the same ecological niche (Hutchinson, 1957), differences in their optimum of environmental conditions are allegeable. However, further research is necessary in order to explain the differences in their susceptibility towards freezing or flooding. More emphasis should be placed on the relations between species-specific behavioural patterns and habitat binding in future studies, as a more detailed knowledge will contribute to our understanding of the ecology immature *Culicoides*.

CHAPTER 8

GENERAL DISCUSSION

The present thesis aimed to answer questions regarding important aspects of the breeding ecology of *Culicoides* with focus on the Obsoletus Group. Overall, these questions could be sufficiently encountered by literature field surveys and experimental studies. A joint discussion and interpretation of the answers found and conclusions drawn will be presented in this last chapter. The first section deals with the methodical requirements of the aspired research (8.1), followed by a summary and interpretation of new information on species-specific breeding ecology and relations to environmental conditions (8.2). The value of the gained knowledge for potential strategies for the control of *Culicoides* will be discussed in section 8.3. Furthermore, the final outlook (8.4) introduces new questions that arose in the scope of this thesis and will point to aspects, which – in the opinion of the author – should be emphasised in future research.

8.1 METHODICAL ASPECTS OF RESEARCH ON *CULICOIDES* BREEDING ECOLOGY

The present thesis placed particular emphasis on the evaluation of methods in research on breeding habitats of *Culicoides*. The obtained information allows a better interpretation of results gained with different study techniques and will facilitate the choice of methods for future investigations. The discussion of results of this thesis repeatedly pointed out that this choice strongly depends on the research aims and the ecology of the species investigated. Therefore, the advantages and disadvantages of the used techniques will hereafter be expounded with regard to different study purposes and the experiences gained in the scope of this thesis. A summary of these aspects is provided in Table 8.1.

One of the key questions asked previously was **how comparable are methods available for the assessment of *Culicoides* abundances in breeding substrates (Question 1)?** Extraction methods sampled significantly higher number of individuals from cowpats, if compared to emergence traps. However, this result must not lead to the perception that flotation or Berlese funnel-extraction are to be preferred on any account. All three methods differ in their adequacy for obtaining certain kinds of information. Thus, prior to a study, the aims and following aspects regarding the investigated habitat and species should be reflected according to the following questions.

- Which life stages are of interest for the study?
- Which life stages are to be expected in the investigated habitat?
- Can immature stages (or adults) be identified morphologically?
- Is the viability of collected individuals relevant?
- Is the density of immature *Culicoides* in the habitat of primary interest?
- Should the natural conditions of the breeding habitat be maintained?
- Is the impact of environmental conditions or treatments on the development relevant?
- How much time and work can be invested?

Extraction techniques, as flotation or Berlese, provide explicit information on colonisation densities of immature *Culicoides* at one time-point. **Sugar-flotation**, in particular can be suggested, if the number of individuals in a breeding substrate shall be determined as exact as possible However, some practice is required in order to produce expedient results, as these also depend on the experience of the researcher and the time spend on the examination of a sample. Flotation methods sufficiently sample *Culicoides* pupae while it must be considered that eggs, and possibly also younger larvae, are too small to be recovered. Thus, Campbell and Kettle (1976) concluded that flotation underestimates *Culicoides* population sizes. This drawback does not apply if only late larvae are targeted, e.g., in overwintering populations of most temperate species.

The **Berlese** extraction can be strongly recommended for testing breeding substrates in advance to a study, if the processing of samples without immature *Culicoides* is to be avoided. Moreover, the technique is time- and work effective and the extraction process can be easily standardised. First larvae from cowpats were usually available within two hours following the start of extraction. Another major advantage is the viability of extracted larvae, providing manifold possibilities for further studies, e.g., on the immature behaviour or the impact of abiotic conditions on the development. This potential was taken advantage of in the experimental studies of this thesis (see chapter 6 and 7). However, one disadvantage of the Berlese has to be taken into account. Pupae can be extracted with this method or be easily reared from extracted larvae, but due to the limited mobility of this life stage, it is probably less appropriate to estimate the number of pupae in a breeding substrate.

A significant tool in *Culicoides* research is the **emergence trap**, which can be applied directly in the field to observe the emergence of *Culicoides* under natural conditions. Minor alterations of the environment, as by shading or reduced ventilation within traps, have to be considered but juvenile stages are not removed from their habitat, as they are for extraction methods. The number of emerged individuals provides a good measure for the development success. This can be utilised to investigate the potential impact of certain conditions on the development, as done in the freezing or flooding experiments described in this work. For an appropriate interpretation of results, it has to be kept in mind that the *Culicoides* density in a substrate might be underestimated using emergence traps. The comparably low trapping success with this method (see section 3.2.4) could be partly due to a natural mortality of immature stages during development. Not all of the eggs or larvae present in a substrate will reach the adult stage and also for extracted larvae or pupae it remains unknown how many would have successfully finished development.

Generally, if results are required within a short time, emergence traps are no ideal choice. Extraction techniques provide results more rapidly. The development of *Culicoides* is highly temperature-dependent (e.g., Akey et al., 1978; Bishop et al., 1996a; Veronesi et al., 2009). Therefore, samples of breeding substrates can alternatively be removed from the habitat and incubated in the laboratory in order to accelerate the development. Zimmer et al. (2013b) preferred this method over magnesium-flotation because of the higher efficiency. However, the removal of substrate involves a more intense alteration of the breeding habitat. Nevertheless, the removal from the field and maintenance of breeding substrates in the laboratory is a considerable alternative to investigate the emergence from dungheaps. Presupposing some mobility of larvae within the dungheap, emergence might not necessarily take place at exactly the same position, at that immature stages previously developed. Thus, to estimate the abundance of immatures in this habitat in relation to the abiotic conditions of the breeding habitat, the maintenance of a defined amount of substrate in the laboratory could produce more accurate results than emergence traps on the dungheap.

In the light of the manifold uses of emergence traps, it is not surprising that the majority of studies mentioned in the literature review on breeding sites of the Obsoletus Group utilised this tool (see 4.1.1.3). Also in the field study of this thesis (see section 4.2), emergence traps were the central method. The sampling design benefited from the minimal effort this method affords, once the traps are installed. Thus, data can be collected in the absence of the researchers. The general significance of emergence traps is indisputable and thus, future research will profit from the optimisation of this tool. Assuming that the lower individual numbers, in comparison to the extraction techniques, are not exclusively due to a natural mortality of immature *Culicoides* during development, a second study aimed to identify factors with impact on the trapping success. More precisely, it was asked **how the trap design, i.e., colour and shape, affects the effectivity of emergence traps (Question 2).** The results support one of the assumptions of the preceding experiment – the low number of individuals can partly be accounted to the fact, that not all emerged *Culicoides* reached the collection container of the traps. As a cause of the positive phototaxis of adult *Culicoides*, dark coloured traps were more effective as compared to white traps. Consequently, it has to be considered, that the abundances, measured with white traps in the field study on cattle farms, might have been underestimations.

Table 8.1 Properties of methods used in research on breeding ecology of *Culicoides*, i.e., Berlese, sugar-flotation and emergence traps used in the field (F) or in the laboratory (L).

	Berlese	**Flotation**	**Emergence trap (F)**	**Emergence trap (L)**
Results refer to	larvae	larvae, pupae	all life stages	all life stages
Sampled life stages	larvae, (pupae)	larvae, pupae	adults	adults
Work load	low	high	low	medium
Processing time	short	short	long	long
Habitat alteration	strong	strong	weak	strong
Advantages	viable larvae	most exact reflection of larval densities	measure for development success, most "natural" conditions	measure for development success, more controlled conditions, can be combined with incubation

Aside from the evaluation of different study methods, it was repeatedly asserted that cowpats provide good possibilities for experimental studies and can be helpful in gaining detailed knowledge on the ecology of dung-breeding *Culicoides*. Cowpats represent clearly definable experimental units that can be easily divided into comparable subsamples. Besides, this sustainable resource is permanently available and easy to transport. For several study purposes, the winter months are preferable over summer or spring: during this season, higher larval densities and higher percentages of colonised cowpats were noticed, compared to the summer. In late summer or autumn, females of *C. chiopterus* or *C. dewulfi* lay their eggs on cowpats on the pastures. Afterwards, no new cowpats are added, as the cattle usually stay inside the stables during winter. Thus, this generation of *Culicoides* is concentrated in a reduced number of available breeding sites.

Also the results of the field study reflect this situation, as the highest number of individuals emerged from cowpats in spring. The good availability of larvae is advantageous, e.g., the extraction of sufficient numbers of larvae for experimental studies is less time-consuming. Moreover, of *Culicoides* species overwintering as larvae, all extracted individuals are at the same life stage.

8.2 NEW INSIGHTS INTO THE BREEDING ECOLOGY OF THE OBSOLETUS GROUP

The present thesis aimed to **identify the main breeding habitats of the species of the Obsoletus Group (Question 3).** Clear breeding site preferences were demonstrated for three of the four species. In the surroundings of cattle farms, dungheaps must be considered as one of the most productive sources of *C. obsoletus* s.s.. In contrast, neither information found in the literature, nor results of the field study pointed to a specific type of breeding habitat for *C. scoticus*. Despite the diversity of substrates this species is able to colonise (see section 4.1.1.2), the numbers of collected individuals were usually low. At the same time, the species is commonly found in light trap catches (Sarto i Monteys and Saiz-Ardanaz, 2003; Baldet et al., 2004; Balczun et al., 2009; Nielsen et al., 2010), leading to the conclusion that the main breeding habitats are either still unknown or that many "minor" habitats contribute to a stable adult population of *C. scoticus*. In both cases, future studies should place stronger emphasis on the investigation of breeding sites apart from livestock holdings, which might serve as reservoirs for *C. scoticus* and other *Culicoides* species.

While *C. obsoletus* s.s. and *C. scoticus* rarely occurred in cowpats, this habitat evidently presents the main developmental site of *C. chiopterus* and *C. dewulfi*. This was evident in field data, experimental studies on cowpats and the literature review. In cowpats, the two species develop in close coexistence in a spatially confined habitat. Two species in the same biocenosis cannot occupy the same ecological niche (Hutchinson, 1957). Thus, differences are to be expected, e.g., in the species optima regarding abiotic conditions or their behaviour. In this context, it does not surprise that *C. chiopterus* and *C. dewulfi* differ in their susceptibility towards environmental changes as freezing or flooding as demonstrated in chapter 6 and chapter 7. Since our knowledge on feeding habits of *Culicoides* larvae is limited, it is also possible that these species utilise different food sources, e.g., different microbe species. The assumption that larvae of the Obsoletus Group rely on the same resources allows a discussion from a different viewpoint: the separation towards different breeding habitats, i.e., the development of *C. obsoletus* s.s. in dungheaps and of *C. chiopterus* and *C. dewulfi* in cowpats, can be explained by means of the competitive exclusion principle excluding the coexistence of two species competing for the same resource (Gause, 1934; Hardin, 1960). If *C. obsoletus* s.s., for example, depends on the same food source as the other two species, competition avoidance would have resulted in the occupation of a different developmental site. This diversification allows the simultaneous development of large numbers of adult midges, and thus, the coexistence of adult populations of these species in the same environment, i.e., the cattle farm.

Apart from the capability of colonising cattle dungheaps in large numbers, *C. obsoletus* s.s. appears quite opportunistic with regard to breeding habitat occupation (see section 4.1.1.1). Two different strategies seem to be pursued by the species of the Obsoletus Group. *Culicoides chiopterus* and *C. dewulfi* can be considered specialists, being almost restricted to cowpats as a breeding substrate. Instead, *C. obsoletus* s.s., and also *C. scoticus*, appear to be generalist species. Against this background, it is without surprise that *C. obsoletus* s.s. is a very successful and ubiquitous species according to light trap studies in Europe (e.g., Conte et al., 2007; Meiswinkel et al., 2008; Balczun et al., 2009). Instead, the abundance of *C. chiopterus* is underestimated with light traps (Carpenter et al., 2008a). Thus, adult densities cannot be sufficiently determined with this method. However, considering their successful development in cowpats, paired with the constant availability of large numbers of these breeding sites and hosts, this species is most likely highly abundant at cattle farms. *Culicoides chiopterus* was assumed to prefer cattle over sheep (Garros et al., 2011). This conformity of larval and adult ecology indicates a close affinity to cattle. In this context, it seems misleading that the results of Carpenter et al. (2008a) refer to a comparison of light trap collections and drop-trap catches on sheep. Against this background, it was suggested that *C. chiopterus* might be attracted to sheep without actually feeding on this host (Garros et al., 2011).

Subsequent to the identification of the main breeding sites of *C. obsoletus* s.s., *C. chiopterus* and *C. dewulfi*, the next question to be answered was, **which physical or chemical factors influence the presence and abundance of the immature *Culicoides* in these habitats (Question 4).** Habitat specialists are generally expected to be stenoecious, due to adaption to the conditions in a certain breeding site. With reference to most abiotic parameters investigated, *C. chiopterus* and *C. dewulfi* cannot be considered stenoecious species, although they are almost restricted to cowpats. These species tolerate a wide range of pH, phosphor concentrations or CN-ratios, however, these tolerances more or less equalled the abiotic conditions measured in cowpats. Some of the environmental parameters measured in the investigated habitats proved helpful in explaining the distribution of immatures in these habitats, as the pH value and the substrate moisture in dungheaps. Comparing these results with information provided in the literature illustrates that conclusions on the impact of abiotic factors need to be considered for species and habitat types separately. The relevance of environmental parameters varies substantially within the genus *Culicoides* and among occupied breeding sites and thus, general statements are futile.

It is evident that livestock holdings, as cattle farms, offer suitable breeding substrates for the species of the Obsoletus Group. This environment, with constant availability of large numbers of hosts, plus suitable breeding sites, provides ideal conditions for the successful development of these haematophagous insects. On cattle pastures, the cows are present in the immediate vicinity of the breeding habitat of *C. chiopterus* and *C. dewulfi*, i.e., cowpats. In stables, large numbers of hosts are gathered in a confined space, often in the immediate vicinity of a particularly productive breeding habitat for *C. obsoletus* s.s., i.e., dungheaps. Moreover, stables seem to provide favourable conditions for *C. obsoletus* s.s.. Adult midges enter buildings and emerge from dung inside stables (Baldet et al., 2008; Meiswinkel et al., 2008; Zimmer et al., 2010; Ninio et al., 2011b).

It is reasonable to expect, that *Culicoides* feeding on livestock, will profit from the accumulation of hosts. Thus, the success of the species of the Obsoletus Group, being widely distributed and abundant in many regions of Europe (Meiswinkel et al., 2008), might be partly related to the recent changes in agricultural structures. Livestock densities have greatly increased in the European countryside (Stoate et al., 2001) and more hosts and associated breeding sites could have resulted in larger *Culicoides* populations. A corresponding example for the relationship between *Culicoides* abundance and agricultural change refers to *C. paraensis*, the vector of the Oropouche virus in the Neotropical region (Tesh, 1994; Felippe-Bauer and Sternheim, 2008). This species develops in decaying plant material. The change of the forest environment to banana plantations in Southern Brazil was suggested to be responsible for the increased incidence of this species, due to the greater availability of breeding sites, i.e., rotting banana stems (Mercer et al., 2003).

Along with increasing livestock densities in Europe in consequence of the agricultural intensification, the overall number of mixed livestock and arable farms strongly decreased (Stoate et al., 2001). Thus, fewer farms provide potential habitats for the Obsoletus Group. If a direct relation exists between *Culicoides* population sizes and the number of hosts and the amount of available breeding sites, respectively, higher densities of Ceratopogonidae are concentrated at fewer locations. Particularly in this scenario, "natural habitats", e.g., forest mud, leaf litter or marshland, distributed in-between such "hotspots", might be of relevance as alternative breeding sites. Presumably, generalist species, as *C. obsoletus* s.s. or *C. scoticus*, also develop successfully in habitats off agricultural environments. Depending on the flight range of *Culicoides*, such habitats could serve as reservoirs for veterinary relevant *Culicoides* species. Mark-release-recapture studies demonstrated flight ranges of several kilometres for *Culicoides*, including upwind movements of up to six kilometres (Brenner et al., 1984; Lillie et al., 1985; Kirkeby et al., 2013). According to a recent study, specimens of the Obsoletus Group can evidently fly up to 2.5 kilometres within 48 hours (Kluiters et al., 2015). Thus, these species would easily reach a cattle farm from a natural reservoir in a few kilometres distance. In addition, passive spread over windborne dispersal is assumed to be possible over much greater distances (reviewed by Elbers et al., 2015). In Belgium, nature reserves were identified as potential reservoirs for veterinary relevant *Culicoides*, e.g., *C. impunctatus*, according to light trapping (Zimmer et al., 2013c). As this study primarily surveyed wetlands, species of the Obsoletus Group were only found in low numbers. Investigations of other potential breeding sites in nature reserves will allow a better assessment of their role as reservoir habitats for these species.

One of the reasons for the great success of the class Insecta is probably the ability to cease life activity during unfavourable conditions and proceed as soon as the environment allows. One example is the survival of hostile temperature, as in northern Europe during winter, where insects have to deal with freezing. The role of the species of the Obsoletus Group as pathogen vectors justifies the following question: **how does freezing of the breeding substrate impact the development success of overwintering larvae (Question 5)?**

While overwintering larvae of species developing in cowpats are confronted with freezing of this habitat, the temperatures in dungheaps can remain high, even in winter (see Manuscript VI, section 11.2.1). However, according to the freezing experiments of this thesis, a part of the larvae of *C. chiopterus* survives temperatures far below the 0 °C in this substrate. Thus, the likelihood for significant reductions of local populations of dung-breeding species by freezing is low. Similar conclusion were made for the overwintering larvae of other species, e.g., for *C. variipennis* in the United States (Jones, 1967). It is not known which strategy allows *Culicoides* to survive such hostile conditions during winter. Quiescence allows insects to cope with short periods of unfavourable conditions. In general, the quiescence is reversible and directly temperature-dependent. Diapause, on the other hand, is a biological strategy allowing species to survive otherwise lethal conditions (e.g., freezing during winter) and is most often induced by photoperiod (Matthews and Matthews, 2010). In the present thesis, larvae of *C. chiopterus* and *C. dewulfi* were very active right after extraction from cowpats in winter and they seemed to immediately precede development. Moreover, daylight rhythms have no impact on the onset of development of overwintering larvae of these species instead of temperature (see Manuscript IX, section 11.2.4). These results do not suggest a winter diapause, at least with regard to *C. chiopterus* and *C. dewulfi*.

One of the consequences of climate change is the ongoing overall increase of temperature, also results in milder winters in Europe (EEA, 2008; IPCC, 2013). In the future, even higher numbers of *Culicoides* might be expected to survive the winter. Research on the responses of *Culicoides* to climate change is just in the initial stage (Elbers et al., 2015), but effects on other insects are already evident. One examples is the disturbed synchrony between the hatching of *Operophtera brumata* L. (winter month) and oak bud burst (Visser and Holleman, 2001), caused by warmer spring temperatures in Europe. Due to anthropogenic activities, such drastic disturbances are not to be expected for dung-breeding *Culicoides* as intensive livestock systems provide ideal conditions for the development during all seasons. It has been argued that the spread of BTV into previously unaffected regions is related to an extension of the northern range limits of *C. imicola*. To date, there is no clear evidence on notable temperature-dependent shifts in the geographic range of *Culicoides* midges, as there is, for example, for certain mosquito species (Mellor et al., 2008; reviewed by Elbers et al., 2015). However, consequences of changing climatic conditions for the distribution, abundance and phenology are to be expected (see Manuscript XI). Increasing temperatures might lengthen *Culicoides* biting seasons and accelerate development cycles – possibly resulting in a higher number of generations per year (Wittmann and Baylis, 2000). This would amount to an even greater challenge regarding the control of vector species.

Another concern of the present thesis was to investigate if **flooding impacts the development success of larvae and pupae of the Obsoletus Group (Question 6).** Besides the increase of temperature, the climate change has also been connected to more frequent and severe floods in Europe (Hall et al., 2014). This trend could impact species of the subgenus *Avaritia* that seem to avoid waterlogged habitats, including the species of the Obsoletus Group. This was pointed out by the field study (see chapter 4) and the literature review on breeding sites (see section 4.1.1). Moreover, the behaviour of *C. chiopterus* and *C. dewulfi* in water (see chapter 7) reflects an adaption to the development in terrestrial habitats. According to this thesis, pupae of these species do not tolerate flooding for longer time periods (i.e., ten days). However, an intensive drainage management minimised the risk for long-term inundation of pastures. In order to allow the optimal agricultural use of lowland areas in Germany, the control of groundwater depths is necessary, i.e., these need to be low enough to allow ploughing in spring. Thus, a reduction of the larval population by flooding can at the most be expected during extreme flood events or along larger rivers in Germany. Here, the faster runoff by intensive drainage was even suggested to have contributed to increased flood frequencies (Krause et al., 2007; reviewed by Hattermann et al., 2013).

8.3 IMPORTANCE OF KNOWLEDGE ON *CULICOIDES* BREEDING ECOLOGY FOR VECTOR CONTROL

In 2007, the Friedrich-Loeffler-Institute (FLI) published a guide with recommendations for farmers regarding the protection of their livestock from *Culicoides*. This guide basically proposed the treatment of livestock with repellents, along with the information that these cannot completely prevent the bites (FLI, 2007). Today, potential control measures for adult *Culicoides* are still being improved, e.g., with research on insecticide-treated netting, the optimisation of repellents or options for biological control (González et al., 2014; Nicholas and McCorkell, 2014; Baker et al., 2015). However, there is still a great need for effective control strategies. Even though Germany was declared BTV-free in 2012 (FLI, 2012), we should maintain permanent awareness of the risk of new introductions of known – or unknown – diseases. The combination of several agents, such as globalisation and climate change, along with the fact that at least 75 viral diseases are associated to *Culicoides* worldwide (Meiswinkel et al., 2004b) contribute to a perpetual threat. As reminders serves the sudden emergence of the Schmallenberg virus in 2011 in Germany and beyond, the recent rise of BTV-4 in southern Europe and the Balkans or the recent reports of new infections with BTV-8 from France (Beer et al., 2013; DEFRA, 2014; DEFRA, 2015; Hornyák et al., 2015). Against this background, it is important to ask: **how can the knowledge gained in the scope of this thesis be applied in *Culicoides* control measures (Question 7)**?

There is an urgent need for an effective control management of *Culicoides* populations. As pointed out in the previous chapter, the likelihood of significant reductions of *Culicoides* populations of the Obsoletus Group by "environmental agents", e.g., freezing in winter or flooding of pastures, is limited. Particularly, in the case of cowpats is an artificial repression of immature populations problematic. The flooding of pastures would require significant effort and moreover disturb other biocenosis on treated meadows. Also the treatment with insecticides or the removal of cowpats, spread over wide areas on pastures, appears an unrealisable task. Moreover, the commonly used pyrethroid-based insecticides are highly toxic for aquatic invertebrates (reviewed by Carpenter et al., 2008b). The application on cowpats would include the risk of their elution by rain and the transport to rivers over agricultural drainage systems. According to previous studies, also the mechanical disturbance of cowpats, by destroying their surface, remained without any significant effect on the emerging population (see Manuscript VIII, section 11.2.3; Künkele, 2013). Instead, treating cowpats with entomopathogenic fungi effectively prevented the development of *C. brevitarsis* (Nicholas and McCorkell, 2014). This might be a promising approach for prospective control measures.

As strongly suggested by the results of this thesis, dungheaps provide a more promising target for control measures. In this habitat – a major source for *C. obsoletus* s.s. – large numbers of immature stages are concentrated in a relatively small area. It is moreover feasible that the temperatures in dungheaps allow the development of *Culicoides* even during the winter months (see Manuscript VI, section 11.2.1). The presence of adult *Culicoides* in this season is of particular importance for the overwintering of viruses and was discussed as one possible reason for the new emergence of the bluetongue epidemic in 2007, after a putative vector-free period exceeding the duration of infectious viremia. Small numbers of infected *Culicoides* might have survived in resting places (Wilson et al., 2008). Specimens of the Obsoletus Group were caught during winter inside of stables in northern Europe and observed to feed on hosts indoors (Losson et al., 2007; EFSA, 2008; Clausen et al., 2009). In combination with a breeding habitat providing suitable temperatures for development during winter, as dungheaps or the dung within stables, complete life cycles appear well possible.

In order to prevent that newly emerged individuals from a dungheap readily reach a near cattle stable, it might be considered to increase the distance between this habitat and the stables. An important requirement for potential control actions is the compatibility with agricultural working processes. In this case, this would probably mean a compromise between a maximal distance, the accessibility of the heap for the farmers and also the distance to surrounding farms. Possibly, the dung could be collected for some time and then be transported to a remote location that could be shared by several farmers. This location should provide no shelter from the wind to additionally aggravate flying activity of *Culicoides.* Also with regard to potential modifications of *Culicoides* habitats on farms, agricultural activities must be considered, e.g., if the dung is used for crop fertilisation, measures should not interfere with rotting processes. Since the presence and abundance of immatures of the

Obsoletus Group decreased inversely with substrate pH in the field study, one potential attempt is the artificial modification of pH in dungheaps. This could be achieved by treatment with lime fertiliser, which is already used to increase the fertility of soil in agriculture. Such treated manure could be spread on the fields allowing the farmer to "kill two birds with one stone". However, according to preliminary studies, the possible increase of the pH through lime seems to be limited. The practicability of this approach needs to be investigated with more effort.

Generally, the most promising time for control measures on breeding sites would be prior to the onset of adult emergence in spring. An effective reduction of immatures would naturally result in a reduction of all following generations, as these individuals could not contribute to the population by means of reproduction. The artificial flooding of dungheaps is one potential strategy. In the field study, immatures of *C. obsoletus* s.s. colonised this habitat up to a height of 2.5 metres. Thus, flooding the bottom of a dungheap would not affect individuals in the higher regions. Therefore, for this purpose, the dung should evenly spread, possibly in a hollow, possibly on tarpaulin or paved underground to minimise the water-runoff. Under these conditions could the dung be artificially inundated by the farmer, in order to prevent a successful development of the immature *Culicoides*. A challenge of this approach would be the right timing. As demonstrated in chapter seven, the pupae of dung-breeding *Culicoides* are more susceptible to flooding than the larvae. Flooding for ten days completely prevented further development. Therefore, this strategy would be most effective if the majority of the immature population were in the pupal stage. Thus, an optimal time would be in spring, as soon as the temperature is high enough to allow the overwintering larvae to continue their development. An exact prediction of this time-point is difficult but the developmental stage could be regularly examined using the Berlese method. A second flooding after a few days would account for variations of the development speed of immatures and thus, increase the effectivity of this measure.

The authors of a study from Great Britain hoped to achieve a reduction of the adult population emerging from dungheaps by covering this habitat with tarpaulins, but simultaneous light trap catches revealed no differences in abundances as compared to untreated farms (Harrup et al., 2014). There are several explanations for the failure of this attempt: the tarpaulin might not have effectively kept the midges from emerging from the dungheap and leaving it. Furthermore, as the present thesis showed that productivity of dungheaps can vary strongly among farms, it is possible that the dungheaps investigated in the British study were simply no productive breeding sites. Thus, control measures with regard to dungheaps will not be equally effective at all sites. Harrup et al. (2014) additionally discussed that breeding habitats in the surroundings of the dungheap might have compensated for a detectable reduction in light trap catches. Indeed, the fact that *C. obsoletus* s.s. can develop in manifold habitats, will hamper control measures. A similar conclusion was drawn during another attempt of *Culicoides* control by habitat modification (Mayo et al., 2014). The waste-water pond of one of two farms in California, serving as a substantial breeding habitat for *C. sonorensis*, was drained. Again, light-trapping did not indicate reduced *Culicoides* abundance at the treated farm, and thus, the authors concluded the waste-water system not to be the sole or primary breeding site. This problematic must also be considered in future control measures targeting dungheaps.

8.4 OUTLOOK

In current research, many studies on *Culicoides* vector species rely solely on the identification with molecular methods, as PCR-based analyses and DNA barcoding (e.g., Augot et al., 2010; Lehmann et al., 2012; Ander et al., 2013; Bellis et al., 2014). It is beyond doubt that the advancements in this domain provide many advantages, as the facilitated separation of morphologically close species – as *C. obsoletus* s.s. and *C. scoticus* – or the time-effective identification of large numbers of specimens. However, despite the challenging and time-consuming aspects of conventional entomological methods, morphological identification skills will never be dispensable. As pointed out by the simultaneous use of morphological and molecular identification methods in chapter 2 (see section 2.3.1), neither the results of molecular, nor of morphological methods should be blindly relied on. Therefore, in future research, more emphasis should be placed on morphological methods to render possible that both kinds of techniques can mutually benefit from each other.

Another methodological aspect of *Culicoides* breeding ecology is the necessity of standardised trapping methods in order to obtain comparable study results. The present thesis provided needful knowledge for the optimisation of emergence trapping. The definition and development of a "standard emergence trap", in collaboration with other scientist of this research field, would be the next reasonable step. Further methodical approaches to be concerned for future investigations should target the oviposition of female *Culicoides*. In initial studies, different types of substrates or replications of the same substrate type varying in one abiotic parameter, e.g., the pH value, could be offered for oviposition. This approach might provide useful information on breeding sites preferences and female behaviour. Ovitraps are widely used in investigations on mosquitoes as the dengue vector *Aedes aegypti* (e.g., Barrera et al., 2014), but are not among the common tools for *Culicoides* research. The development of suitable ovitraps for *Culicoides* vector species would provide better options for studies on species distribution and ecology. Moreover, it would facilitate the sampling of eggs (as an alternative to the sampling of eggs from wild-caught females), and thus provide better possibilities for the rearing of laboratory colonies and experimental studies on all life stages.

The knowledge on many aspects of the ecology of immature stages of *Culicoides* is still poor, e.g., at several points of this thesis, questions on larval food sources of the Obsoletus Group species arose. A better knowledge, e.g., through the analysis of gut contents, could explain habitat preferences and provide starting points for the disturbance of pre-adult development. Wide knowledge gaps in immature taxonomy and morphology additionally hamper the research on juvenile life stages. It was recently summarised, that among the described *Culicoides* species only 3% are known as eggs, 13% as larvae and 17% as pupae (Borkent, 2014). Therefore, an increase of studies identifying and describing the immature stages of this genus is needed, in particular for veterinary relevant species.

Today, there is still a great need for studies incorporating vector control experimentally (reviewed by Mullens et al., 2015). Future research should also place particular emphasis on experimental studies, e.g., to assess the impact of environmental parameters on the development success of vector species. The studies on the impact of freezing and flooding (see chapter 6 and 7) have contributed to a better knowledge in this field. However, further studies are necessary, e.g., experimental freezing treatments, which reflect "natural" conditions, e.g., by temperature fluctuations according to day-and-night rhythms. The results of chapter 7 pointed out that the larvae of *C. chiopterus* and *C. dewulfi* were more tolerant towards flooding as compared to the pupae. This gives rise to the question if and to what extent the juvenile stages would sustain the opposite environmental extreme, i.e., desiccation or heating. Moreover, the abundance of immature *C. obsoletus* s.s. is affected by substrate pH and moisture. Clearly defined lethal thresholds of these parameters would allow a better utilisation of this knowledge for potential control measures.

The experimental studies on *C. chiopterus* and *C. dewulfi* provided valuable knowledge on the biology of these species but analogue information regarding *C. obsoletus* s.s. and *C. scoticus* could not be obtained. Large numbers of larvae of the first two species were easily attained by extraction via Berlese. This method successfully identified colonised cowpats for studying. Preliminary attempts to extract *C. obsoletus* s.s. larvae from dungheap samples were of limited success, possibly due to the variability of this breeding habitat. Therefore, the potential of the Berlese-extraction for the screening of dungheap samples and other potential habitats should be emphasised in the future. Obtaining larvae of *C. scoticus* is particularly difficult as the main breeding sites of this species are not obvious. Thus, prospective field studies should search for potential habitats of this species outside of agricultural environments. A better access to immature stages of the Obsoletus complex would allow the implementation of experimental studies, as successfully conducted for *C. chiopterus* and *C. dewulfi*, for *C. obsoletus* s.s. and *C. scoticus*. Of course, according studies on vector species aside from the Obsoletus Group, e.g., *C. imicola* or *C. pulicaris*, would also provide valuable information.

The present study was mainly focused on the investigations of cattle holdings. Dairy farming is more intensively done in Germany, in comparison to sheep farming. Nevertheless, sheep are susceptible to BTV and the Schmallenberg virus (Darpel et al., 2007) and are known as hosts for *Culicoides*, including the species of the Obsoletus Group (see section 1.2). About 1.6 million sheep were maintained in livestock holdings in 2015 in Germany (Statistisches Bundesamt, 2015). Therefore, future research on breeding sites should also address potential habitats on local sheep farms. Application possibilities of the gained knowledge for the control of *Culicoides* in the surroundings of cattle farms were discussed in the previous section, pointing out, that the suppression of *Culicoides* emergence from cowpats represents a particularly challenging task. A similar conclusion was already drawn in 1966 during investigations on another species developing in cowpats, i.e., *C. brevitarsis* (Cannon and Reye, 1966). This problematic underlines the need of a close collaboration with farmers in order to develop management strategies that are incorporable in agricultural processes. The combination of their expertise with the knowledge and experiences from *Culicoides* research might lead to new approaches for pest management. As a start, the Berlese method could be introduced to farmers. This method is easy to apply and would provide good possibilities for regular screening of suspected breeding habitats on site. At the current state of knowledge, the temporary flooding of dungheaps, prior to the onset of *Culicoides* emergence in spring, appears a most promising management approach. The potential of this idea should be tested in experimental studies, ideally in consultation with farmers.

Generally, a combination of different methods appears most reasonable in order to successfully prevent the emergence of diseases transmitted by *Culicoides.* The protection of cattle from being bitten (as by protective housing) and the reduction of adult *Culicoides* by targeting the immature population should go along with a regular adult surveillance. The latter is essential to assess the risk of potential virus transmission (primarily depending on the presence of adult midges) and to diagnose the success of control measures. Moreover, any reluctance of reporting potential infections of livestock must be dispelled by political means, in order to confine the risk of further disease spreading. Hence, farmers should be granted financial support in the case of an outbreak. In this context, a constant awareness of clinical symptoms of the bluetongue or Schmallenberg disease must be aspired. The effective control of *Culicoides* is a particular challenging task. This statement was valid nine years ago (FLI, 2007) and is still true today. However, a profound knowledge on the breeding sites and the ecology of *Culicoides* will considerably increase our chances of successfully bearing this challenge.

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10. SUMMARY

10.1 SUMMARY (ENGLISH)

The thesis investigates several aspects of the breeding ecology of the four *Culicoides* species of the Obsoletus Group, i.e., *C. obsoletus* s.s., *C. scoticus*, *C. chiopterus* and *C. dewulfi*. These species are among the main vectors of viruses affecting ruminant livestock, as the bluetongue or the Schmallenberg virus. The knowledge on the immature stages of the genus, their breeding sites and development is generally poor, however, essential for the development of effective control measures. The main aims of the thesis included the evaluation and improvement of methods for the research on breeding ecology of *Culicoides*, and the identification of important breeding habitats of the Obsoletus Group at and around cattle farms in Germany. Moreover, the potential influence of abiotic conditions on the distribution of the immature stages in identified developmental substrates was investigated. The impact of freezing and flooding on the development success was further assessed in experimental studies.

1. In the comparison of emergence traps, Berlese funnel-extraction and sugar-flotation for the assessment of abundances of immature *Culicoides* in cowpats, significantly more individuals were sampled with the first two methods. The Berlese method provides many benefits, e.g., the rapid availability of data and extraction of viable larvae. However, the choice of method generally depends on the study purpose, the ecology of the investigated species and the type of breeding substrate.
2. Comparing different emergence trap designs, the trap shape (cone-shaped or quadratic) had no impact on the trapping success, while black coloured traps sampled *Culicoides* emerging from cowpats more effectively than white traps. This difference is presumably related to the stronger light gradient between trap body and collection container in black traps and the positive phototaxis of adult *Culicoides*. Thus, an ideal trap design should maximise this light gradient, by using an opaque or reflecting trap material, and allow sufficient aeration in order to reduce a potential heat-up within the trap.

3. The most productive breeding habitat of *C. obsoletus* s.s. in the surroundings of cattle farms are dungheaps, while *C. chiopterus* and *C. dewulfi* develop primarily in cowpats. No typical habitat of *C. scoticus* could be identified.
4. A decreasing substrate pH was associated with a higher abundance or a higher probability of observing *C. obsoletus* s.s. in dungheaps, as well as *C. chiopterus* and *C. dewulfi* in cowpats. Furthermore, the abundance of *C. obsoletus* s.s. was positively affected by increasing moisture and decreasing trap height on dungheaps.
5. In the exposure of cowpat subsamples to freezing for 48 hours at different sub-zero temperature levels, no immature *Culicoides* survived freezing at -21 °C. In contrast to *C. dewulfi*, a small number of *C. chiopterus* developed after freezing at -18 °C. In combination with a temperature monitoring within cowpats on a pasture, the results indicated, that a significant reduction of the immature populations of these species by low temperatures during winter is unlikely.
6. In experimental floodings of immature stages of *C. dewulfi* and *C. chiopterus*, larvae were neither able to swim nor were the pupae able to float. Flooding for 24 hours did hardly affect larvae and pupae, but an exposure for 10 days killed nearly all larvae of *C. dewulfi*, 50% of *C. chiopterus* and all pupae of both species. These observations most likely indicate a strong adaption to the development in terrestrial habitats, i.e. cowpats.
7. In consideration of the present results, potential control measures should target the overwintering larval population in breeding habitats, prior to the onset of adult emergence in spring. Dungheaps provide good preconditions for treatments of breeding substrates, as large numbers of immature *C. obsoletus* s.s. can be found in this habitat in a comparably confined space.

10.2 ZUSAMMENFASSUNG (DEUTSCH)

Im Rahmen dieser Doktorarbeit wurden verschiedene Aspekte der Brutökologie der Obsoletus Gruppe untersucht. Die vier Arten dieser Gruppe, *C. obsoletus* s.s., *C. scoticus*, *C. chiopterus* und *C. dewulfi* gehören zu den Hauptvektoren von Viren, die schwerwiegende Krankheiten bei Wiederkäuern hervorrufen, z.B. das Blauzungen- oder das Schmallenberg-Virus. Über die juvenilen Stadien, ihre Brutplätze und ihre Entwicklung ist generell wenig bekannt, jedoch ist ein umfassendes Wissen für den effizienten Einsatz von Bekämpfungsmaßnahmen essentiell.
Die Hauptziele der Arbeit umfassten die Evaluierung und Optimierung von Untersuchungsmethoden für die Erforschung der Brutökologie von *Culicoides*, sowie die Identifizierung wichtiger Bruthabitate der Obsoletus Gruppe im Umfeld von landwirtschaftlichen Betrieben mit Rinderhaltung. Darüber hinaus wurde der Einfluss verschiedener abiotischer Parameter auf das Vorkommen der Entwicklungsstadien in identifizierten Bruthabitaten untersucht. In experimentellen Studien wurden Auswirkungen von Frost und Überflutung auf den Entwicklungserfolg einbezogen.

1. Beim Vergleich von Emergenzfallen, Berlese und Flotation für die Bestimmung der Abundanz juveniler *Culicoides* in Kuhdung, wurden mehr Individuen mit den ersten zwei Methoden gefangen. Die Berlese-Methode erwies sich als besonders vorteilhaft (schnelle Datenverfügbarkeit, Lebensfähigkeit extrahierter Larven), jedoch hängt letztendlich die Wahl der Methode stark vom Zweck der jeweiligen Studie, der Ökologie der untersuchten Arten und der Art des Brutsubstrates.
2. Im Vergleich verschiedener Emergenzfallen-Typen hatte die äußere Form der Falle keinen Einfluss auf den Fangerfolg, während mit schwarzen Fallen mehr Individuen als mit weißen Fallen gefangen wurden. Der Unterschied ist wohl auf den stärkeren Lichtgradienten in dunklen Fallen und die positive Phototaxis der adulten Gnitzen zurückzuführen. Ein ideales Fallendesign sollte daher den Lichtgradienten maximieren, z.B. durch die Verwendung eines blickdichten oder reflektierenden Materials, und gleichzeitig eine ausreichende Ventilation ermöglichen, um einem Temperaturanstieg in der Falle entgegenzuwirken.
3. Die wichtigsten Bruthabitate für *C. obsoletus* s.s. auf landwirtschaftlichen Betrieben mit Rinderhaltung sind Misthaufen, während *C. chiopterus* und *C. dewulfi* sich primär

in Kuhfladen entwickeln. Ein typisches Habitat für die Entwicklung von *C. scoticus* wurde nicht identifiziert.

4. In Misthaufen und Kuhfladen stieg mit abnehmendem pH-Wert die Abundanz und die Wahrscheinlichkeit für das Auftreten von *C. obsoletus* s.s., *C. chiopterus* und *C. dewulfi*. Die Abundanz von *C. obsoletus* s.s. in Misthaufen war darüber hinaus positiv von einem hohen Feuchtigkeitsgehalt beeinflusst. Die höchsten Individuenzahlen wurden in bodennahen Fallen und in Fallen in mittlerer Höhe auf dem Misthaufen gefangen.
5. Beim Einfrieren von Kuhfladen für 48 Stunden bei unterschiedlichen Temperaturen überlebte ein Teil der überwinternden Larven von *C. chiopterus*, aber nicht von *C. dewulfi*, das Einfrieren bei -18 °C. Nach dem Einfrieren bei -21 °C entwickelten sich keine juvenilen Stadien weiter, während Imagines beider Arten zahlreich aus den Kontrollproben schlüpften. Diese Ergebnisse und die Resultate einer Überwachung der Temperatur in Kuhfladen auf einer Rinderweide während der Wintermonate belegen dass eine signifikante Reduktion der überwinternden Populationen dieser beiden Arten durch Frost im Winter unwahrscheinlich ist.
6. Bei der experimentellen Inkubation juveniler Stadien von *C. dewulfi* und *C. chiopterus* in Wasser sind bei beiden Arten die Larven nicht in der Lage zu schwimmen und die Puppen nicht zum Auftrieb im Wasser fähig. Eine Überflutung für 24 Stunden beeinträchtigte die Entwicklung von Larven und Puppen kaum. Eine Exposition von 10 Tagen tötete fast alle Larven von *C. dewulfi* and 50% von *C. chiopterus* ab sowie alle Puppen beider Arten. Diese Beobachtungen deuten auf eine starke Anpassung an die Entwicklung in terrestrischen Lebensräumen hin.
7. Aufgrund der vorliegenden Ergebnisse wird empfohlen, dass potentielle Bekämpfungsmaßnahmen die überwinternde Larvenpopulation in Bruthabitaten fokussieren, und zwar im Vorfeld der einsetzenden Emergenz im Frühjahr. Misthaufen stellen ideale Ziele für Maßnahmen dar, da die Larven von *C. obsoletus* s.s. in diesem Habitat in großer Menge auf kleinem Raum zu finden sind.

11.1 MANUSCRIPTS: FIRST-AUTHORED

11.1.1 MANUSCRIPT I

Assessment of the abundance of *Culicoides chiopterus* and *Culicoides dewulfi* in bovine dung: A comparison of larvae extraction techniques and emergence traps

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Assessment of the abundance of *Culicoides chiopterus* and *Culicoides dewulfi* in bovine dung: A comparison of larvae extraction techniques and emergence traps

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ABSTRACT

We compared two larvae extraction methods involving sugar-flotation and an adapted Berlese funnel-extraction with emergence traps. This was done in order to analyse the colonisation of cowpats by *Culicoides chiopterus* (Meigen 1830) and *Culicoides dewulfi* (Goetghebuer 1936) (Diptera: Ceratopogonidae) and to gain information on the comparability and efficacy of these three methods. With all three methods, a considerable number of individuals was obtained. Significantly more individuals were obtained via sugar-flotation and Berlese funnel-extraction compared to the emergence traps. These differences, likely due to natural mortality and sample processing are discussed. We recommend Berlese as an efficacious method for extracting *Culicoides* larvae from bovine dung. It produces data rapidly and extracted larvae are viable. In comparison with Berlese, slightly more larvae were obtained by sugar-flotation, but this method was very labour intensive.

1. Introduction

Biting midges of the genus *Culicoides* are vectors of several veterinary important arboviruses, including African horse sickness virus, bluetongue virus and Schmallenberg virus (Mellor et al., 2000; Rasmussen et al., 2012). The immature stages of different species of this genus develop in a variety of habitats, including tree holes (Murray, 1957), intertidal sand (Jamnback and Wall, 1958), bogland, swamps and animal dung (Kettle and Lawson, 1952). Consequently, diverse techniques are available for the assessment of individual numbers in different development substrates. These can basically be assigned to two main strategies: one can either try to extract and count the larvae or pupae or wait for emergence to count the adult midges.

A variety of methods have been used to extract immature stages of *Culicoides* species from different substrates, e.g. sieving, flotation, Berlese funnel-extraction or agar-extraction (reviewed by Hribar, 1990). The applicability is dependent on the ecology and behaviour of the species involved. For *Culicoides* species developing in waterlogged habitats, it can be sufficient to float samples with water and collect the swimming larvae (Carpenter, 1951). Other methods depend on the active migration of the larvae by swimming into a new medium, which facilitates collection (Dove et al., 1932; Boreham, 1981). To extract larvae that cannot swim, flotation techniques relying on solutions with a high specific gravity are useful options (organic material floats to the solution surface while inorganic material settles at the bottom). Different kinds of suspension have been successfully used, e.g. in solutions with sugar (Dyce

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and Murray, 1966; Blanton and Wirth, 1979; Blackwell and King, 1997), magnesium sulphate (Ladell, 1936; Kettle and Lawson, 1952; Wirth, 1952; Zimmer et al., 2013) or sodium chloride (Mullens and Rodriguez, 1984). However, no standard method is available to extract the larvae of veterinary important vector species.

An alternative to larvae extraction is the work with emerged adult midges. Emergence traps are commonly used in current research concerning the ecology of immature *Culicoides* (Kirkeby et al., 2009; Foxi and Delrio, 2010; Gonzalez et al., 2013; Harrup et al., 2013; Thompson et al., 2013). A variety of designs have been applied in numerous studies, including recovery cages made from wooden boxes (Dove et al., 1932), tent-like emergence traps of white netting (Pajor, 1987), plastic buckets (Uslu and Dik, 2010) and conical shaped traps of tarred roofing paper (Braverman, 1970) or black cardboard (Dyce and Marshall, 1989; Bishop et al., 1996a, 1996b, 2005).

Although different larvae extraction techniques have been compared (Bidlingmayer, 1961; Mullens and Rodriguez, 1984), there is not much information available on how the efficiency of these methods compare to that of emergence traps. Kline et al. (1975) compared sieve-, sand- and magnesium sulphate flotation with Berlese funnel-extraction and rearing chambers and showed that the efficiency of these methods varies among species. However, most individuals were obtained with sieve- and salt-flotation. On the other hand, Campbell and Kettle (1976) obtained fewer individuals of the dung breeder *Culicoides brevitarsis* by magnesium sulphate flotation compared to emergence traps. Nevertheless, information on the relation between extraction techniques and emergence traps for *Culicoides* biting midges is limited. Therefore, the main objective of the study was to compare the efficiency of two different larvae extraction techniques to that of emergence traps to determine the abundance of the immature stages of *Culicoides chiopterus* and *Culicoides dewulfi* in cowpats. Both these species are potentially involved in the transmission of bluetongue virus (Meiswinkel et al., 2007; Dijkstra et al., 2008) and Schmallenberg virus (De Regge et al., 2012, Elbers et al., 2013) and use cow dung as a larval medium (Kettle and Lawson, 1952; Campbell and Pelham-Clinton, 1960). Since the larvae of *C. chiopterus* and *C. dewulfi* cannot swim, flotation with water or other methods dependent on this ability would not be sufficient. Therefore, sugar-flotation, considered to be less harmful to the larvae than salt-flotation (Hribar, 1990), was compared to an adapted Berlese funnel-extraction method. Several authors have used Berlese- or Tullgren-funnels in order to recover *Culicoides* larvae (Jamnback and Wirth, 1963; Jamnback, 1965; Kline et al., 1975; Blanton and Wirth, 1979; Blackwell et al., 1999) or larvae of the subfamily *Forcipomyia* (Saunders, 1959). According to Hribar (1990), it is a useful method to extract larvae from several substrates, such as rotting leaves, mosses or aquatic plants. However, it is not known if the method extracts immature stages successfully from animal dung.

Studies of development sites can provide valuable information on the ecology of *Culicoides*. Therefore, we investigated the relation and comparability of emergence traps and extraction methods. The study further examines the applicability of such methods to bovine dung, which serves as a development substrate for *Culicoides* species of veterinary importance.

2. Methods

2.1. Preliminary study: effect of manual mixing of manure samples on the larval distribution pattern and emergence

For a meaningful comparison of flotation, Berlese and emergence traps, uniformity among the cowpat subsamples compared was necessary. It cannot be assumed that immature *Culicoides* are evenly distributed within cowpats (Bishop et al., 1996a). It was, therefore, determined if manual mixing of the cowpat material results in an even distribution of larvae and if this change of substrate structure will negatively affect the number of individuals emerging. Cowpats for the preliminary tests were collected on a dairy farm in northwestern Germany (November, 2013) on a pasture approximately 160 m from a cow shed. Five cowpats were divided into two equal-sized halves. One of the halves was thoroughly mixed and blended by hand. With 200 g of this mixture, a new cowpat was formed. From the other half a triangular piece (reaching from the edge to the middle of the cowpat to integrate material from the inner and outer pat regions) of approximately 200 g was cut out. This procedure was repeated for all five cowpats resulting in five mixed and five unmixed subsamples of equal weights. These were placed under emergence traps, composed of a reversed plastic bucket (5.9 L) with a transparent top collection container (Fig. 1). The container was filled with approximately 50 ml of salt solution (200 g/L), supplemented by a drop of detergent. Emerging insects fly into the top container and are preserved in the salt solution. Every second to third day samples were moistened with tap water (approximately 20 ml per sample) and all emerged

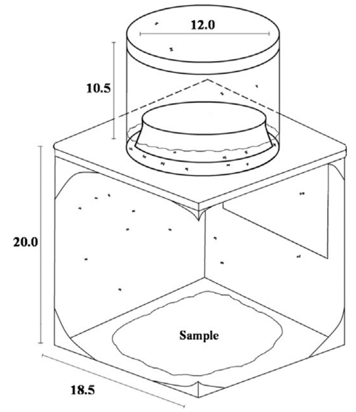

Fig. 1. Schematic drawing of the applied emergence trap, composed of a reversed plastic bucket (5.9 L). Two lateral aeration windows are covered with gauze (mesh size: 500 μm). A hole at the top of the trap leads to a transparent collection flask, filled with approximately 50 ml of salt solution (200 g/L) to catch emerging insects.
Source: Adapted from A. Wittmann.

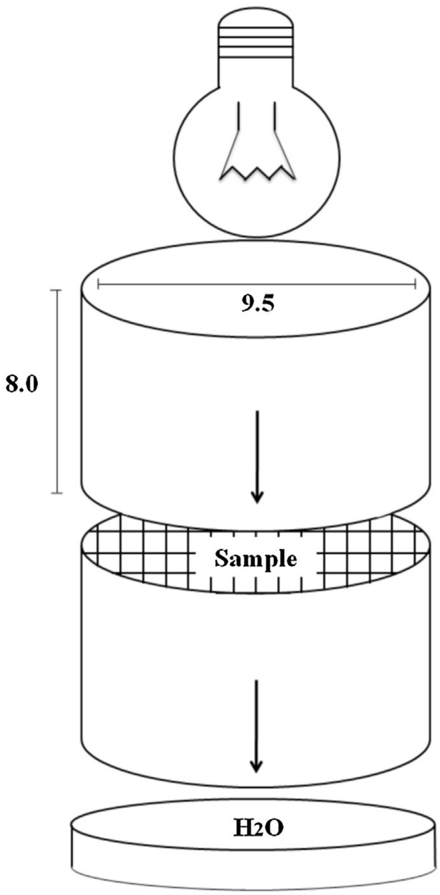

Fig. 2. Schematic drawing of the applied Berlese device. A coarse meshed gauze (mesh size: 0.5 cm) is fixed between two plastic cylinders. The sample is spread on the gauze. The cylinders are set upon a petri dish filled with tap water (distance sample to water surface: ~6 cm) and a halogen lamp (57 W) is placed above (distance sample to light source ~ 10.7 cm).

insects were collected out of the salt solution with a pipette. Adult *Culicoides* were sorted out and counted. The numbers of emerged individuals from mixed and unmixed samples were statistically compared with a Wilcoxon signed-rank test using the program R (R Development Core Team, 2011).

In a second test, the material of one cowpat was mixed, as described above, and divided into 12 subsamples (50 g each). A modified Berlese-Tullgren device was used to evaluate the uniform distribution of *Culicoides* larvae between these subsamples, referred to as "Berlese" within the present work (Fig. 2). The dung was spread on coarse-meshed gauze fixed between two plastic cylinders, into a layer approximately 1-cm thick. Cylinders were used instead of funnels to avoid the danger of larvae sticking to the funnel neck. The cylinders were set upon a petri dish filled with tap water, and a halogen lamp (57 W) was placed above. Larvae migrating downwards fell into the water in the petri dish. *Culicoides* larvae were sorted out and counted daily. Afterwards, this procedure was repeated with two additional cowpats with six subsamples (50 g each) from each pat.

2.2. Method comparison: Berlese, sugar-flotation and emergence traps

In January of 2014, ten cowpats were collected on the same dairy farm mentioned above (see method section of preliminary study). As the study aimed at the overwintering biting midge population, the cowpats were collected from a pasture on which the last grazing by cows had taken place about four months earlier. The samples were transported to the laboratory and each pat was thoroughly mixed as described before. From every cowpat, 100 g of the mixed material was placed in a Berlese device (see method section of preliminary study). The temperature within the samples was monitored with miniature data loggers (iButton DS1921G, Maxim Integrated, San Jose, CA, USA). *Culicoides* larvae were sampled from the petri dishes every 24 h until no *Culicoides* larvae were found for two consecutive days. The water within the petri dishes was replaced daily. Five moderately colonised cowpats were used for the comparison of the three methods, i.e. Berlese, emergence traps and sugar-flotation. A second subsample of 100 g was taken from every cowpat, placed in the Berlese device and processed the same way as the first sample, resulting in a total amount of 200 g from each cowpat tested. The sugar-flotation method was tested with 200 g from the same five cowpats. Subsamples of 20 g were rinsed over a sieve cascade (sieves: 2-mm and 63-μm mesh size). The content of the last sieve (63-μm mesh size) was mixed in a dish (24.5 cm × 10 cm) with 100 ml of a saturated sugar solution. Afterwards, the samples were screened using a stereo microscope at low magnification (10×), and *Culicoides* larvae were counted and preserved in alcohol. Finally, 200 g of each of the five cowpats was placed under emergence traps (see method section of preliminary study) at a rearing temperature of 20.3 ± 1.4 °C. Trapped insects were removed every second or third day, and cowpat samples were moistened with tap water (approximately 20 ml per sample). This procedure was continued until no *Culicoides* biting midges were trapped for 14 consecutive days. The *Culicoides* larvae extracted with the sugar-flotation and Berlese methods were identified to species level according to the key of Kettle and Lawson (1952). To verify the identification of these larvae, 257 individuals collected via Berlese were reared to adults. These individuals and *Culicoides* biting midges collected with emergence traps were identified following the key of Campbell and Pelham-Clinton (1960).

Two weeks after the first method comparison, the entire procedure was repeated; ten cowpats were collected and tested with the Berlese method and five 'positive' samples (i.e. cowpats containing *Culicoides* larvae) were chosen, resulting in a total of ten cowpats used for the comparison of Berlese, sugar-flotation and emergence traps.

2.3. Statistical analysis

All statistical analyses were carried out with the program R (R Development Core Team, 2011). Confidence levels of 5% were used to define statistically significant differences. We compared the results of the three methods by performing a one-way repeated measures ANOVA with the fixed factor METHOD and the

Table 1
Number of *Culicoides* larvae obtained via Berlese from subsamples (50 g) of thoroughly mixed cowpat material. Twelve subsamples from cowpat 1 were used, and six subsamples were used from cowpats 2 and 3.

Subsample	Cowpat 1	Cowpat 2	Cowpat 3
1	28	14	23
2	32	16	26
3	33	17	28
4	40	19	31
5	40	22	32
6	41	23	35
7	41		
8	44		
9	47		
10	48		
11	49		
12	53		

random factor PAT for *C. chiopterus* and *C. dewulfi* separately. Paired *t*-tests were applied to compare the results obtained with the individual methods with each other. Furthermore, Spearman's rho rank correlation coefficient (ρ) was calculated using the function "corr.test" from the R-package "psych" (Revelle, 2011) for all possible pairwise comparisons (emergence/flotation, emergence/Berlese, flotation/Berlese). *P*-values obtained from *t*-tests and correlation coefficients were corrected after Bonferroni to take multiple comparisons into account.

3. Results

3.1. Preliminary study: effect of manual mixing of manure samples on larval distribution patterns and emergence

A total of 1296 *Culicoides* emerged from the mixed cowpat samples (mean ± SE: 259 ± 155.9), and a total of 1136 individuals emerged from the unmixed samples (227 ± 91.6). The numbers of emerged individuals from the mixed and unmixed treatments were not significantly different (Wilcoxon signed-rank test, $n = 5$, $p = 0.63$). The relatively low standard errors of the results of the second preliminary study (Berlese with mixed subsamples) indicate an even distribution of larvae within the samples: 41 ± 7 *Culicoides* larvae were obtained from the first cowpat ($n = 12$, min. = 28, max. = 53), 19 ± 4 larvae from the second cowpat ($n = 6$, min. = 14, max. = 23), and 29 ± 4 ($n = 6$, min. = 23, max. = 35) from the third cowpat (Table 1).

3.2. Method comparison: Berlese, sugar-flotation and emergence traps

From all ten cowpats, larvae were recovered with the two larvae extraction techniques, and adult *Culicoides* biting midges were obtained with the emergence traps. In total, 1898 *Culicoides* larvae were obtained via sugar-flotation (Table 2). The majority of *Culicoides* larvae were identified as either *C. chiopterus* (59.7%) or *C. dewulfi* (39.8%), except for ten larvae not assignable to any of the species due to poor condition of the individuals (0.5%). These ten larvae were excluded from further analysis. On average, 22 ± 5 min were needed to float 20 g of cowpat material. The Berlese method yielded 1575 larvae (Table 2). Of these individuals, 64.2% were identified as *C. chiopterus* and 35.8% as *C. dewulfi*. Larvae were detected for up to four days from the start of the experiment, but the majority of individuals were recovered during the first 48 h (95.4%). During the Berlese method, the temperature in the samples ranged from 23.6 ± 0.7 °C during the first 24 h to 26.0 ± 3.5 °C on the fourth day. The species differentiation of larvae (following the key of Kettle and Lawson, 1952) and reared adults (following the key of Campbell and Pelham-Clinton, 1960) was in accordance in all cases. Of the *C. chiopterus* larvae, 71.1% (of 180 larvae), and of the *C. dewulfi* larvae, 98.7% (of 77 larvae) were reared successfully.

With the emergence traps 1117 adult *Culicoides* were collected. Of these individuals, 66.0% were identified as *C. chiopterus* and 33.7% as *C. dewulfi* (Table 2). One female individual was identified as *C. obsoletus* and was excluded from further analysis as along with four individuals not assignable to any species due to bad material condition

Table 2
Total number of larvae and mean number of larvae in cowpat sub samples of 200 g (±standard deviation) yielded with sugar-flotation and Berlese and total number and mean number (±standard deviation) of adults obtained with emergence traps.

Method	Sugar-flotation	Berlese	Emergence trap
Total nr. of *C. ch.*	1133	1011	737
Mean nr. of *C. ch.*	113.3 ± 65.9^{A}	101.1 ± 50.3^{A}	73.7 ± 53.0^{B}
Total nr. of *C. de.*	765	564	376
Mean nr. of *C. de.*	75.6 ± 84.2^{A}	56.4 ± 57.2^{A}	37.6 ± 44.1^{B}
Ratio *C. ch.*:*C. de.*			
Sample 1	2.2	3.2	1.9
Sample 2	5.5	2.8	6.5
Sample 3	0.7	1.7	0.9
Sample 4	3.3	3.0	2.8
Sample 5	2.0	2.1	1.3
Sample 6	1.9	1.5	15.7
Sample 7	4.7	4.7	40.0
Sample 8	4.4	5.5	14.6
Sample 9	0.1	0.2	0.2
Sample 10	4.1	4.7	4.6
Mean	2.8 ± 1.8	2.8 ± 1.6	8.2 ± 11.8

The numbers per row followed by a different letter were significantly different at the 5% level. Further, the ratio (number of *C. chiopterus* individuals divided by number of *C. dewulfi* individuals) of *C. chiopterus* (*C. ch.*) to *C. dewulfi* (*C. de.*) for the ten cowpats is given.

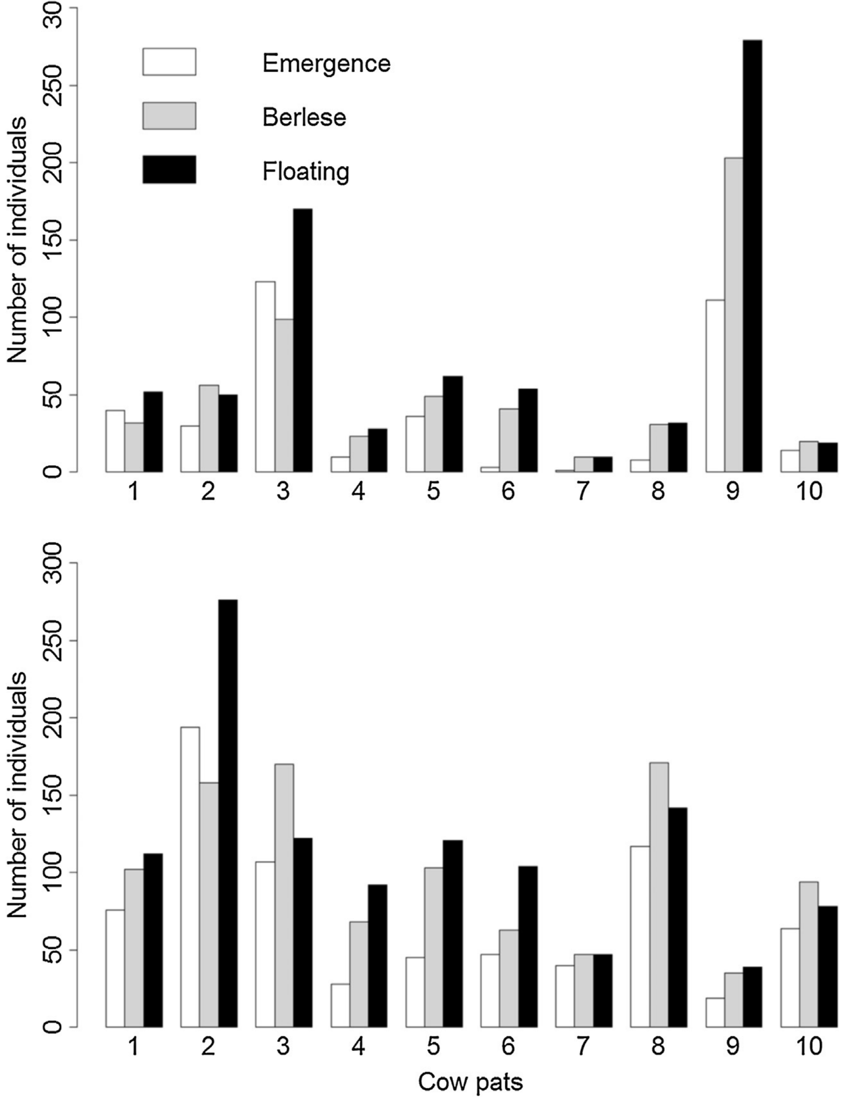

Fig. 3. Number of larvae, respective adult individuals of *C. dewulfi* (top) and *C. chiopterus* (bottom) obtained via sugar-flotation, Berlese and emergence traps from subsamples of ten cowpats.

(0.4%). The sex ratio (number of males divided by the number of females) was 1.17 for *C. chiopterus* and 1.03 for *C. dewulfi*. The experiment was stopped when no *Culicoides* were found in the traps for two consecutive weeks. This was the case after 73 days (first experimental unit) and 59 days (second experimental unit).

In general, sugar-flotation yielded more individuals than Berlese and the least number of individuals was found in the emergence traps (Fig. 3). On average, 73.7 ± 53.0 adult *C. chiopterus* were trapped in the emergence traps, 101.1 ± 50.3 *C. chiopterus* larvae were extracted via Berlese and 113.3 ± 65.9 larvae of this species were sampled via sugar-flotation. Accordingly, 37.6 ± 44.1 adult *C. dewulfi* were trapped in the emergence traps, and 56.4 ± 57.2 larvae were recovered via Berlese. Calculation of Spearman's rho rank correlation coefficient (ρ) revealed positive correlations between the number of *C. chiopterus* individuals obtained by Berlese/flotation ($n = 10$, $\rho = 0.92$, $p < 0.001$), Berlese/emergence ($n = 10$, $\rho = 0.84$, $p < 0.01$) and flotation/emergence ($n = 10$, $\rho = 0.85$, $p < 0.01$). For *C. dewulfi*, positive correlations occurred between Berlese/flotation ($n = 10$, $\rho = 0.93$, $p < 0.001$), Berlese/emergence ($n = 10$, $\rho = 0.75$, $p < 0.05$) and flotation/emergence ($n = 10$, $\rho = 0.73$, $p < 0.05$). The percentage of larvae extracted via Berlese, exceeded the number of adults sampled with emergence traps for both, *C. chiopterus* (57.5 ± 49.2% more individuals via Berlese compared to emergence traps) and *C. dewulfi* (279.3 ± 441.2% more individuals via Berlese compared to emergence traps) whereas the percentage of larvae extracted via flotation compared to Berlese was 15.2 ± 34.4% higher in *C. chiopterus* and 23.9 ± 28.0% higher in *C. dewulfi*. The one-way repeated measures ANOVA showed a significant effect of the applied method for both species: *C. chiopterus* ($F\,(2.9) = 6.7$, $p < 0.01$) and *C. dewulfi*

(F (2.9) = 5.2, $p < 0.05$). The paired t-tests revealed significant differences of the number of extracted larvae/emerged adults between sugar-flotation and emergence traps (paired t-test, $t = 4.50$, df = 9, $p < 0.01$) and between Berlese and emergence traps (paired t-test, $t = 2.95$, df = 9, $p < 0.05$) for *C. chiopterus*. Comparing flotation and Berlese, the ratios of *C. chiopterus* to *C. dewulfi* within samples from the same cowpats were generally similar. A comparison of species ratios from the larvae extraction techniques and emergence traps showed a stronger variation (Table 2).

4. Discussion

Various methods are available to determine the abundance of *Culicoides* larvae in development substrates, but there is not much information available on the relative effectiveness of these methods. Therefore, we conducted a comparison of the performance of emergence traps, sugar-flotation and Berlese.

A considerable number of *C. chiopterus* and *C. dewulfi* individuals was obtained with all three methods. However, there were clear differences in the effectiveness. Significantly more individuals were obtained using the extraction techniques compared to the number of trapped adult midges in the emergence traps. This pattern is in accordance with the results of Kline et al. (1975) who obtained more individuals of *Culicoides furens*, *Culicoides hollensis* and *Culicoides melleus* from salt marsh soil with sieve- and salt flotation compared to rearing chambers. There are several possible explanations for the lower individual numbers we obtained with the emergence traps compared to the extraction techniques. It cannot be excluded that more biting midges emerged, but they were not caught in the collection flask of the traps. Moreover, while the numbers of adult midges obtained in the emergence traps will have been influenced by the natural mortality of the immature stages during development, this will not have influenced the numbers of larvae obtained with the larvae extraction methods. Also hibernation processes have to be considered. However, as rearing success from larvae extracted with the Berlese was very high, it is unlikely that larvae within the cowpats in the emergence traps did not develop due to diapause. Nevertheless, the developmental success might have been different under natural conditions in the field. Even though larvae were reared in substrates from their natural habitat, the transfer of samples from outside to the moderately tempered laboratory could have affected the development of immature stages.

In contrast to the present results, Campbell and Kettle (1976) concluded that flotation underestimates the total population, as *Culicoides* eggs are too small to be recovered. Undoubtedly, this has to be considered when different developmental stages are expected in the studied substrate. However, it does not apply to our study, as our sampling design aimed at larvae overwintering within the cowpats. The presence of only one developmental stage is an advantage for a comparison of methods, as younger instars or eggs would have affected the results obtained with emergence traps only, providing that these stages are not detected with extraction methods. The larvae extracted via Berlese or flotation, were approximately the same size. The larvae developed into adult midges in a short time (10 ± 3.7 days, data not shown). Thus, it can be suggested that they were in the fourth instar when extracted, which is the overwintering life stage for most *Culicoides* species in northern Europe (Kettle, 1984; Szadziewski et al., 1997). As a consequence, the results of the present study merely apply to the late larvae instars and provide no information on the effectiveness for younger instars or pupae.

Generally, all methods tested were quite applicable to cow dung. The somewhat higher number of larvae obtained with sugar-flotation was not statistically different from that obtained with the Berlese method. Apart from this result, flotation was much more labour intensive, and results obtained with this method are likely to be affected by the experience of the operator and the time spent on examination of a sample. The findings of the study suggest that the Berlese approach is most appropriate for *Culicoides* larvae extraction from cowpats. It can be used to survey large numbers of samples in a comparably short time and can be easily standardised for light intensity, sample amount and processing time. Moreover, for studies requiring the emergence of adult *Culicoides* midges from development sites, we recommend testing potential substrates in advance with the Berlese method to avoid working with samples without immature *Culicoides*. Contrarily, Kline et al. (1975) considered Berlese funnel-extraction insufficient for *C. furens* and *C. hollensis* when comparing various extraction techniques with soil samples. However, the adequacy of the method depends on the species and substrate the method is used for (e.g., capability of movement within the respective substrate). Furthermore, while we worked with sample layers of 1 cm and observed migration within four days, Kline et al. (1975) used the technique with sample layers of 2.5 cm and worked the Berlese for 48 h. It is possible the samples did not dry out fast enough to drive the larvae to migrate within this time.

A further advantage of the Berlese technique is the good viability of extracted larvae collected in tap water, which allows successful rearing to adults. Preliminary attempts to rear adult *Culicoides* biting midges from larvae recovered via sugar-flotation were unsuccessful (data not shown). Instead, Cannon and Reye (1966), who used a combination of water flotation and magnesium sulphate flotation to extract larvae of *C. brevitarsis* from cow dung (method adopted from Bidlingmayer, 1961), were able to successfully rear larvae. However, rearing success was low (41%) compared to the results we obtained with larvae extracted via the Berlese method.

To address the danger of a patchy distribution of immature stages within cowpat samples, Bishop et al. (1996a) proposed to take core samples from defined positions in the centre and outside regions of the pats. A clear advantage of this method is the reduced alteration of the investigated substrate, but due to the varying shape and height of cowpats this approach might be difficult to standardise. Our method changes the structure of sample material but facilitates the comparison of subsamples from the same cowpat. The comparison of numbers of emerged individuals from mixed and unmixed cowpat samples (preliminary tests) did not indicate a negative influence of substrate alteration by manual mixing. However, it cannot be excluded that the change of substrate structure might affect the development of other developmental stages, such as *Culicoides* pupae or eggs. Nevertheless, larvae were relatively uniformly distributed after mixing of the substrate, as shown by the preliminary study. Moreover, the individual numbers obtained from subsamples of the same cowpats during the method comparison correlated strongly between the two larvae extraction methods, i.e. if high numbers of larvae were obtained from a cowpat with the flotation method, comparably high numbers were also found in a subsample of the same cowpat using Berlese. These results and the similarities in species ratios of *C. chiopterus* to *C. dewulfi* within subsamples from the same cowpats (Table 2) indicate an even distribution of larvae in subsamples and an equally efficacious performance of the extraction methods for *C. chiopterus* and *C. dewulfi*, whereas the individual numbers obtained via emergence traps showed a greater variability in comparison.

To understand the breeding ecology of *Culicoides* biting midges, comparable study methods are necessary. A lot of current research focuses on data collection with light traps. However, the development of management strategies cannot solely depend on adult population studies. Immature stages represent a large proportion of biting midge populations, while light trap catches most likely reflect abundances of host-seeking females. Further, not all species are trapped with equal success; Carpenter et al. (2008) have shown that abundances of *C. chiopterus* are underestimated by light trap catches. A comprehensive knowledge of the development sites of relevant *Culicoides* species can contribute to risk assessment and help to specify control measures (Carpenter et al., 2008).

5. Conclusion

According to our comparative analysis of methods, larvae extraction techniques yielded more individuals compared to emergence traps. The Berlese method should be favoured over sugar-flotation when dealing with cow dung, as it is much less labour intensive. Furthermore, information on individual numbers is available within a short time. It is especially recommendable when viable larvae are needed for subsequent rearing. Nevertheless, the preferred method will depend on the purpose of the study and the ecology of the species involved. Larvae extraction techniques such as flotation or Berlese do not account for sublethal effects in experimental studies or natural mortality of immature stages, but give explicit information on the colonisation density at specific time points. The three methods tested are well comparable and applicable for cow dung. For other common development substrates, the efficiency and comparability of the methods have to be analysed in future studies.

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11.1.2 MANUSCRIPT II

Comparison of emergence traps of different colour and shape in trapping of *Culicoides* (Diptera: Ceratopogonidae)

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Title

Comparison of emergence traps of different colour and shape in the trapping of *Culicoides* (Diptera: Ceratopogonidae)

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Keywords

C. chiopterus; *C. dewulfi*; cowpat; development; temperature; trap design; vector

Abstract

Different types of emergence traps are available for investigations of the breeding habitats of *Culicoides* (Diptera: Ceratopogonidae). In order to assess the potential impact of the trap design on the trapping success, we compared the efficiency of white- and black-coloured emergence traps and two trap shapes (cone-shaped and quadratic), to sample *Culicoides* emerging from cowpats. Significantly higher numbers of *C. chiopterus* and *C. dewulfi* were trapped with black traps, while there was no obvious effect of the trap shape. There were no distinct differences in the microclimate among different trap types.

1. Introduction

The haematophagous biting midges of the genus *Culicoides* transmit several arboviruses to livestock, e.g., bluetongue virus, Schmallenberg virus and African horse sickness virus (Mellor et al., 2000; Rasmussen et al., 2012). The immature stages of this genus occupy a variety of breeding sites, e.g., moorland, tree holes and animal dung (Kettle and Lawson, 1952; Murray, 1957). Emergence traps are a common method in research on *Culicoides* breeding ecology. A variety of different trap designs have been applied in previous studies to sample emerging *Culicoides*, e.g., constructions of wooden boxes and tarred roofing paper (Dove et al., 1932; Davies, 1966; Braverman, 1970). Further designs include tent-like traps of white netting, plastic buckets, black cardboard cones and simple ice cream containers (Battle and Turner, 1972; Pajor, 1987; Dyce and Marshall, 1989; Bishop et al., 1996; Uslu and Dik, 2010).

It is not known whether different trap designs are comparably efficient in trapping *Culicoides*. Therefore, the aim of the present study was to evaluate the effectivity of four different trap designs. In order to estimate the potential impact of the trap shape on the trapping success, we compared cone-shaped and quadratic designs. To facilitate sampling, emergence traps are often used in combination with transparent collection containers in order to utilise the positive phototaxis of *Culicoides* (Becker, 1960; Bidlingmayer, 1961; Megahed, 1956). A dark or non-transparent trap body maximises the differences in light intensity between trap and collection beaker. It was therefore tested whether a dark trap colour increases the number of trapped *Culicoides* compared to white-coloured traps. The advantages and disadvantages of the tested trap types are discussed in comparison with alternative types proposed in previous studies.

2. Materials and methods

2.1 *Culicoides* sampling

The sampling took place in June 2014 on the pasture of an organic cattle farm in north-western Germany (GPS coordinates: N53 9.985 E8 8.771). Two days in advance of the main experiment, small subsamples (approximately 10 g each) were collected from the edges of 25 cowpats for the extraction of *Culicoides* larvae with an adapted Berlese device (see Steinke et al., 2014 for details) to ensure that only cowpats containing immature stages of *Culicoides* were included in the experiment. Ten colonised cowpats were randomly selected and sampled as a whole from the pasture along with the soil layer (down to 3–4 cm) underneath. A sample of each cowpat (14x14 cm) was divided into four subsamples, which were placed into four different emergence traps: (i) cone-shaped and white-coloured, (ii) cone-shaped and black-coloured, (iii) quadratic and white-coloured, and (iv) quadratic and black-coloured (Fig. 1). Cone-shaped traps were constructed from reversed plastic funnels and quadratic traps from reversed plastic buckets. The traps were combined with transparent collection containers at the top (material: polystyrene, e.g., LICEFA, Germany, Bad Salzuflen) and sealed with a closing plate at the bottom (Fig. 1). Both trap types had two lateral aeration windows (10x10 cm) covered with gauze (mesh size: 105 µm). The collection container was filled with approximately 50 ml of 20% NaCl saline, supplemented by a drop of detergent. Ten traps of each shape were masked with a non-transparent black foil for the black-coloured trap versions. Data loggers (HOBO Pro v2, Onset, Bourne, MA, USA), fastened inside 12 emergence traps (three loggers per trap type), measured air temperature and humidity at hourly intervals. Additionally, miniature temperature loggers (iButton DS1921G, Maxim Integrated, Sunnyvale, CA, USA), carefully inserted into the centre of the 12 respective cowpat subsamples, recorded the substrate temperature. A weather station at a distance of 1.2 km from the test site (Oldenburg University, www.uni-oldenburg.de/wetter) provided information on the ambient temperature.

The traps were placed on a mowed lawn (without noteworthy sources of shadow) and secured with tent pegs for stabilisation. The light intensity at the bottom of all the traps was measured once during the experiment (12–3 pm, 7 August 2014) with a lux meter (PeakTech® 5025, PeakTech, Ahrensburg, Germany). Every second to third day, trapped insects were removed and the cowpat samples moistened with 20 ml of tap water using a spray diffuser. *Culicoides* biting midges were identified to the species level following the key of Campbell and Pelham-Clinton (1960). The experiment was stopped after 47 days, subsequent to two weeks without observed emergence of *Culicoides*.

2.2 Statistical analysis

All statistical analyses were carried out with the program R (R Core Team, 2014) using a confidence level of 5%. The relation between the number of trapped individuals (all *Culicoides*, *Culicoides chiopterus* (Meigen, 1830) and *C. dewulfi* (Goetghebuer, 1936) and the colour and shape of the emergence traps was investigated by applying linear mixed-effects models (function *lme*, package "nlme", version 3.1-120, Pinheiro et al., 2015). The ID of each cowpat was included as a random effect ("pat") in the model to take variations of the *Culicoides* abundance in different cowpats into account. After respective exclusion of a fixed effect, i.e., "colour" or "shape", the original and the new model were compared via ANOVA. The models were run with and without outliers to see whether the exclusions led to distinct changes in *p*-values or coefficient estimates. For evaluation of the final models, a conditional coefficient of determination (R^2) was calculated (Nakagawa and Schielzeth, 2013; Johnson, 2014) and residual plots were produced (histograms of Pearson residuals, fitted values vs. Pearson residuals) for model evaluation.

3. Results and discussion

A total number of 665 *Culicoides* midges were collected, i.e., 464 specimens of *C. chiopterus* (179 males, 285 females) and 201 *C. dewulfi* (33 males, 168 females). The total numbers of trapped *Culicoides* varied greatly among cowpats from six individuals from all subsamples of one pat to 179 midges (66.5 ± 50.6). With quadratic white-coloured traps, 70 *Culicoides* specimens (7.0 ± 5.7) were trapped (Fig. 2), while 101 individuals (10.1 ± 9.0) were collected with cone-shaped white-coloured traps. Overall, more individuals were trapped with the black traps, i.e., 318 (31.8 ± 29.8) with the quadratic trap and 176 (17.6 ± 21.5) with the cone-shaped trap.

According to regression analysis, a significantly higher number of trapped *Culicoides* and *C. chiopterus* were correlated with the black-coloured traps whereas the shape did not significantly affect the number of captured midges (Table 1). For *C. chiopterus*, dropping of the fixed effect "shape" led to bad model fit (patterns in residual plots) and it was therefore retained in the model. For *C. dewulfi*, analysis of the full data set indicated a significant effect of "colour", but no effect of shape. However, the exclusion of two outliers in the response resulted in this variable being non-significant.

Temperature and relative humidity were very similar within different trap types (Table 3.5). The ambient temperature and relative air humidity were on average 4.5 ± 7.0 °C and 10.8 ±

1.7 %, respectively, lower than the average data within traps. The light intensity was more than three times higher in white traps than in black traps (Table 2). In the comparison of different trap designs the trap shape did not affect the number of captured dung-breeding *Culicoides*. However, higher numbers were collected in black traps, presumably caused by a better attraction to the collection container by the differences in light intensity. The differences cannot be due to a temperature-dependent retarded development because the temperatures in both types of traps and also in the cowpat samples were very similar. Assuming that comparable numbers of individuals emerged from subsamples of the same cowpats, fewer midges were trapped in the collection container at the top of the white traps. No accumulation of dead midges was observed inside the traps, but dried-up individuals of insects of such a small size as *Culicoides* are probably hardly visible on the substrate after more than six weeks.

According to the present results, an adequate trap design should include a black or opaque trap body to maximise the differences in light intensity between the trap and the collection container. Especially in warm-climate regions, the risk of a heat-up within the traps must be minimised, because the development of immature *Culicoides* is affected by temperature (e.g., Akey et al., 1978; Bishop et al., 1996; Veronesi et al., 2009). In the present study, the heat-up of air temperature in the emergence traps was moderate. The cooling effect by evaporation of the catching saline and sample moisture in combination with the ventilation through the aeration windows might have compensated for potential overheating. Since the use of air-permeable material could reduce the heat-up within traps, trap bodies made from white gauze are recommended (Pajor, 1987). An optimal trap design should allow for sufficient aeration and address the positive phototaxis of adult *Culicoides* with a strong light gradient between the trap body and the collection container. Prospective studies might consider reflecting or opaque and permeable material for trap construction.

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Table 1 Regression coefficient estimates (± standard errors), standard deviation (SD) of the random effect “pat” and *p*-values for fixed effects retained in the linear mixed-effects models (*lme*) analysing the relation between trapped *Culicoides* and the colour and shape of emergence traps. The number of analysed observations (n), Akaike’s information criterion (AIC) and the conditional coefficient of determination (R^2) for the final variable sets are given

Response	Model coefficients	Estimate	*p*-value	SD	AIC	R^2
i. No. of trapped *Culicoides*	colour: white	-16.2 ± 5.4	< 0.01		344.7	0.3
	pat (random effect)			9.3		
ii. No. of trapped *C. chiopterus*	colour: white	-10.0 ± 3.8	< 0.05		312.3	0.2
	shape: quadratic	5.1 ± 3.7				
	pat (random effect)			3.1		
iii. No. of trapped *C. dewulfi*	intercept	-1.36 ± 0.30	< 0.001		196.5	0.6
	pat (random effect)			8.4		

Table 2 Minimal (min), mean (± standard deviation) and maximal (max) temperature (°C), relative humidity (%) and the mean light intensity (lx) measured outside of traps and at the inner wall within the four trap types

		Temperature (°C)			Relative humidity (%)			Light intensity (lx)
		min	mean	max	min	mean	max	mean
Ambient		9.4	18.6 ± 4.3	31.3	24.1	66.2 ± 18.8	96.1	77035 ± 23702
Cone-black	Air	7.2	23.5 ± 10.4	57.1	20.9	78.5 ± 21.3	100	5500 ± 6189
	Pat	9.7	21.9 ± 6.7	42.9				
Cone-white	Air	7.1	23.7 ± 10.8	59.3	9.4	74.6 ± 22.8	100	20250 ± 4119
	Pat	9.2	23.0 ± 7.9	52.7				
Quadratic-black	Air	7.3	22.8 ± 9.4	52.9	24.7	76.8 ± 18.8	100	4180 ± 901
	Pat	8.8	21.8 ± 6.7	47.3				
Quadratic-white	Air	7.4	22.5 ± 9.3	51.8	19.9	77.9 ± 18.8	99.7	14140 ± 1704
	Pat	8.8	21.9 ± 6.9	45.6				

Fig. 1 Schematic drawing of the quadratic (top left) and cone-shaped trap (bottom left). Aeration windows (A) are covered with gauze (mesh size: 105 µm). Emerging insects are trapped and preserved in the salt solution in the transparent collection container (B).

Fig. 2 Number of trapped *Culicoides*, *C. chiopterus* and *C. dewulfi* specimens with cone-shaped black traps (cb), cone-shaped white traps (cw), quadratic black traps (qb) and quadratic white traps (qw).

Figure1

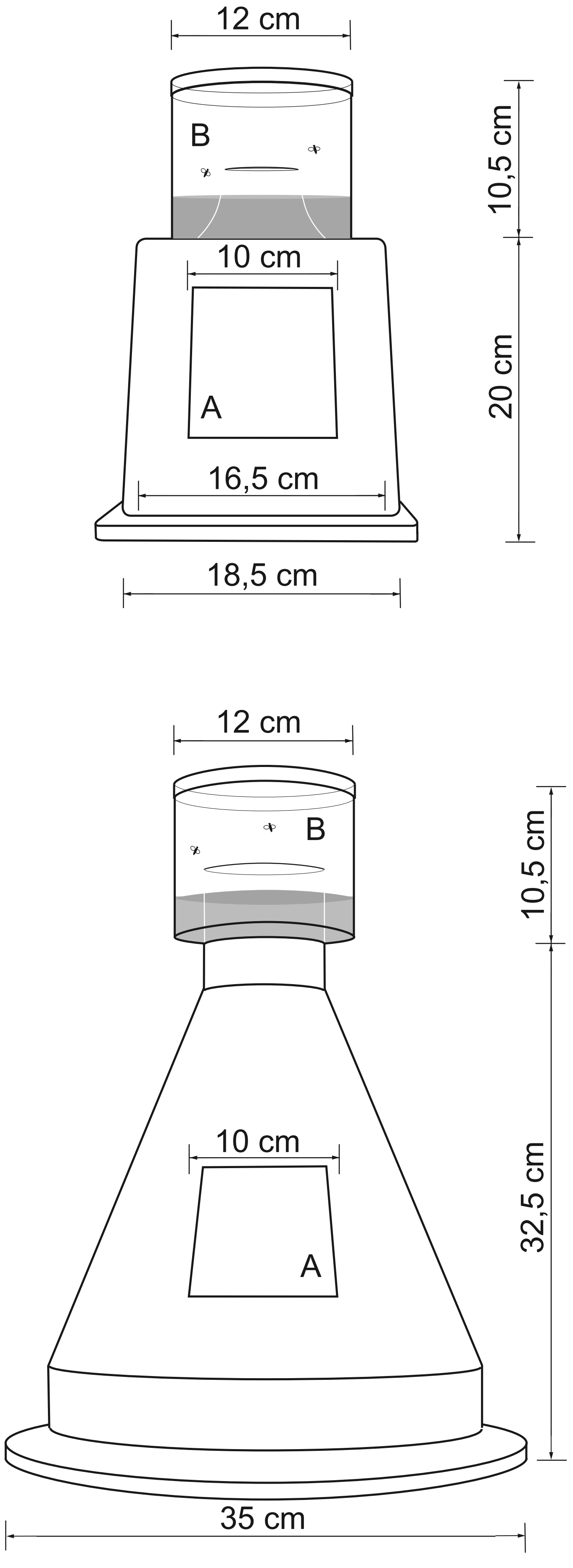
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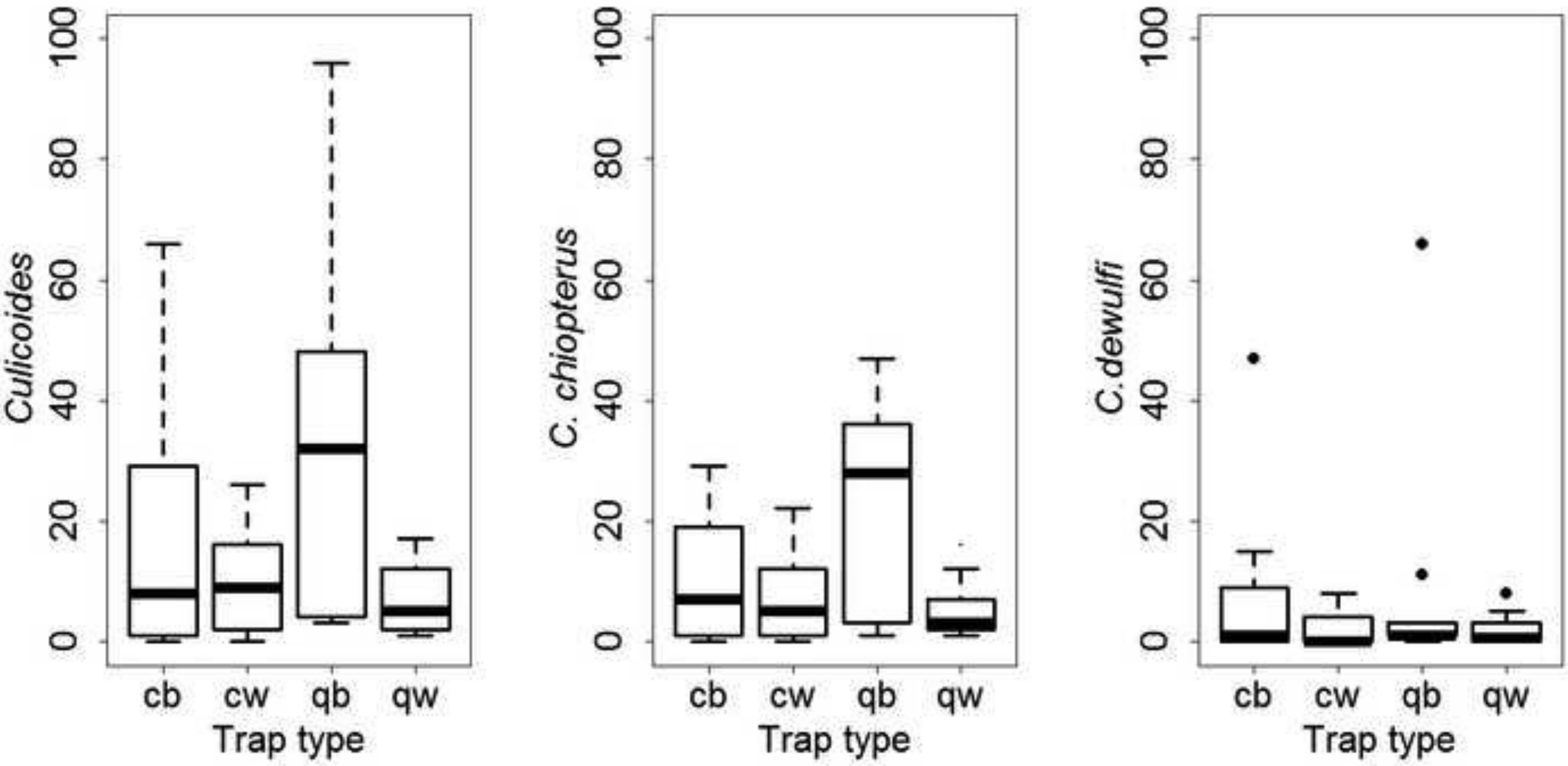
Figure 2
Click here to download high resolution image



11.1.3 MANUSCRIPT III

Emergence of the *Culicoides obsoletus* group species from farm-associated habitats in Germany

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Medical and Veterinary Entomology (in press)

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Emergence of *Culicoides obsoletus* group species from farm-associated habitats in Germany

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Abstract. Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) may transmit several arboviruses to ruminant livestock. The species of the Obsoletus group are considered to be among the most important vectors of bluetongue virus (BTV) in northern Europe. As agricultural environments offer suitable habitats for the development of their immature stages, the emergence of adult *Culicoides* from potential breeding sites was investigated at 20 cattle farms throughout Germany in 2012 and 2013. In analyses of species-specific habitat preferences and relationships between *Culicoides* abundance in breeding substrates and their physicochemical characteristics, dungheaps emerged as the most important substrate for the development of *Culicoides obsoletus* sensu stricto (s.s.) (Meigen), whereas *Culicoides chiopterus* (Meigen) and *Culicoides dewulfi* Goetghebuer were generally restricted to cowpats. A decreasing pH value was associated with a higher abundance or a higher probability of observing these three species. Furthermore, the abundance of *C. obsoletus* s.s. was positively related to increasing moisture. Dungheaps were very productive breeding sites for this species and are therefore suggested as a target for potential control measures.

Key words. *Culicoides chiopterus*, *Culicoides dewulfi*, *Culicoides obsoletus* s.s., *Culicoides scoticus*, cowpat, development, dungheap, larvae, moisture, pH, sex ratio.

Introduction

Culicoides are small biting flies belonging to the family Ceratopogonidae (Diptera). About 1400 species have been identified worldwide (Borkent, 2015). The females of most species feed on the blood of a wide range of hosts, such as cattle, horses, sheep, deer, pigs, rabbits and birds (Koch & Axtell, 1979; Bartsch *et al.*, 2009; Ninio *et al.*, 2011a). Many culicoids are pests to humans; for example, *Culicoides impunctatus* Goetghebuer is a severe problem for tourism in western Scotland (Blackwell & Page, 2003). Far more problematic is their role as vectors to livestock of pathogens such as BTV, Schmallenberg virus and African horse sickness virus (Mellor *et al.*, 2000; Rasmussen *et al.*, 2012). The bluetongue epidemic that occurred during 2006–2008 in northern and central Europe caused massive losses among farmed ruminants. The financial impact of this period in Germany has been estimated to amount to €254 million (Conraths *et al.*, 2012). Bluetongue disease mainly affects cattle and sheep, causing abortion, malformation of calves or lambs, or fever (Osburn, 1994; Elbers *et al.*, 2008). In 2007, 13.1% of affected cattle and 41.5% of affected sheep died as a consequence of the disease in Germany (Conraths *et al.*, 2009).

Consequently, research focused on the identification of species of *Culicoides* associated with ruminant livestock. According to these investigations, species of the Obsoletus group are very important vectors of BTV in northern Europe (Meiswinkel *et al.*, 2008a; Carpenter *et al.*, 2009; Hoffmann *et al.*, 2009). The group comprises four species: *Culicoides obsoletus* s.s.; *Culicoides scoticus* Downes and Kettle; *C. chiopterus*, and *C. dewulfi*.

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However, the inclusion of the latter species is considered controversial (Schwenkenbecher *et al.*, 2009).

The majority of investigations of *Culicoides* are based on data gained with blacklight suction traps, which sample adult individuals. A disadvantage of this method is that only a fraction of the population is represented as males are not equally attracted (Venter *et al.*, 2009). In addition, abundances of particular species are underestimated using such traps, as demonstrated for *C. chiopterus* (Carpenter *et al.*, 2008). Therefore, research on breeding ecology is an important supplement to studies of adult *Culicoides*. The larval stage has the longest duration in the lifecycle (Kettle, 1962) and therefore provides good opportunities for the imposition of control measures. Some species of *Culicoides* develop in specific habitat types [e.g. the intertidal sand species *Culicoides melleus* (Coquillett) (Linley & Mook, 1975)], whereas other species can develop in a variety of substrates. For the development of the immature stages of the Obsoletus group, agricultural environments offer suitable substrates, such as cowpats, silage residues, and animal litter in stables and dungheaps (Kettle & Lawson, 1952; Zimmer *et al.*, 2008; Ninio *et al.*, 2011b; Harrup *et al.*, 2013). However, *C. obsoletus* s.s. occupies various breeding sites, such as the muddy edges of springs, clayey soil, rotting leaves, marshland, tree holes and garden compost (Edwards *et al.*, 1939; Hill, 1947; Kettle & Lawson, 1952; Murray, 1957; Campbell & Pelham-Clinton, 1960; Kremer, 1965).

Recent studies have provided information on livestock-associated breeding sites of *Culicoides* from Spain, southeast England, Northern Ireland and Belgium (González *et al.*, 2013; Harrup *et al.*, 2013; Thompson *et al.*, 2013; Zimmer *et al.*, 2013, 2014). The present survey investigated potential breeding habitats at 20 cattle farms throughout Germany. The major aims of the study were to analyse habitat preferences in species of the Obsoletus group, and relationships between *Culicoides* abundance and the physicochemical characteristics of the investigated substrates.

Materials and methods

Breeding sites

The study was conducted on 19 cattle farms distributed throughout Germany (Fig. 1). One of the farms had to be replaced after the first sampling in 2012, resulting in a total of 20 farms. Sampling was conducted during four periods: August and September 2012; April and May 2013; June 2013, and July and August 2013. During each period, up to 10 emergence traps per farm were set up on potential breeding substrates for 14 consecutive days. The emergence traps were constructed of reversed white plastic funnels (32 cm high, covering 0.08 m^2) with two lateral aeration windows covered with gauze (mesh size: 105 μm), and a transparent top collection container filled with a preservation liquid [aqua dest : ethanol (96%) : glycerine = 4 : 3 : 2, plus a drop of detergent] [see Lühken *et al.* (2014a) for trap figure and further details]. A total of 229 traps were placed on dungheaps (total number of all traps on dungheaps, at all farms and during all sampling periods). Further habitat types investigated were cowpats (225 traps), water-associated substrates (241 traps), bark mulch (23 traps) and compost (11 traps). The authors attempted to select cowpats that were at least a few days old by considering the consistency of the pat and the height of the grass around the cowpat. Older cowpats were also sampled. However, a reliable judgement of age was not possible. During April and May 2013, mainly cowpats from the previous year were investigated in order to target the overwintering *Culicoides* population. Traps on water-associated substrates were usually placed upon the moist sediment adjacent to the waterline, the bank or the centre if drying up of the water bodies had occurred. This habitat type comprised ditches (90 traps), ponds (74 traps), puddles (31 traps), leakage from silage, dungheaps or slurry (22 traps), reed filtration systems (11 traps), rain drainage (seven traps) and bogland (six traps). A sample of each substrate was taken for soil analysis in the laboratory (sample of the substrate under the emergence trap down to a depth of 10 cm) in order to measure the concentrations of potassium and phosphate, pH value, the amount of organic matter (percentage, the difference between dry weight and ash-free dry weight) and the ratio of carbon to nitrogen (CN ratio) with a CHNS analyser (Flash 2000; Thermo Fisher Scientific GmbH, Braunschweig, Germany).

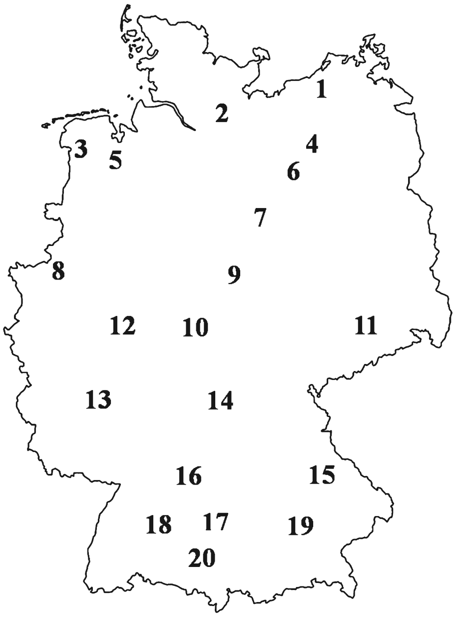

Fig. 1. Locations of the 20 cattle farms in Germany. Farm 20 was included only in 2012 and farm 17 only in 2013.

In addition, substrate moisture was measured on site in soft or diluted substrates (e.g. dungheaps) with a moisture probe (HD2 in combination with a Trime®-Pico 64/32; IMKO GmbH, Ettlingen, Germany). Where this method was not applicable (in firmer substrates or cowpats), three standardised substrate samples (150 cm^3 per sample) were taken (because of their small size, cowpats usually supplied enough material for one sample). These samples were weighed on site (fresh weight) and dried in the laboratory in order to determine the dry weight and water content (difference in weight between fresh and dry substrate).

Furthermore, the (potential) amount of shade at sample sites was estimated (never shaded, temporary shaded, constantly shaded), taking into account objects that cast shadow in the vicinity of the trap, and the height of the emergence traps on dungheaps was measured [distance from the bottom rim of the trap to the ground, ranging from 0 m (standing on the ground) to 4 m]. The position of each trap was determined using a portable GPS device (GPSMap 62S; Garmin Deutschland GmbH, Garching, Germany).

Culicoides identification

Trapped insects were removed after the sampling period of 14 days. Biting midges of the genus *Culicoides* were preserved in ethanol (70%). Species of the Obsoletus group (i.e. *C. obsoletus* s.s., *C. scoticus*, *C. chiopterus* and *C. dewulfi*) were identified morphologically following the key and remarks of Campbell & Pelham-Clinton (1960), by comparing the distinctness of wing pattern, the form of the third segment of the maxillary palp and the number of hairs on the first tergite. As the differentiation of female *C. obsoletus* s.s. and *C. scoticus*, according to morphological features, is prone to error, these were not identified to species level and instead were classified as *C. obsoletus* s.s./*C. scoticus*.

Statistical analysis

All statistical analyses were carried out in the program R (R Core Team, 2014) using a confidence level of 5% to define statistically significant differences. Several tools are available in R for the regression analysis of count data. Thus, different approaches to the analysis of the relationship between *Culicoides* emerging from breeding sites and the abiotic parameters of the respective substrates were considered, including generalized linear models (overdispersion corrected for with quasi-glm), zero-altered negative binomial models and zero-inflated negative binomial models [functions *hurdle* and *zeroinfl*, package PSCL Version 1.4.9 (Zeileis *et al.*, 2008)]. The present data were characterized by excess zeros (absence of the Obsoletus group in 70% of all samples) and overdispersion, caused by large count data and the large amount of zeros. In particular, zero-altered negative binomial models and zero-inflated negative binomial models are useful methods for handling these issues (Zeileis *et al.*, 2008). However, residual analysis of the results indicated that the models did not explain the present data sufficiently. Therefore, a two-step approach was adopted. In the first step, a linear mixed-effects model [function *lme*, in package NLME Version 3.1-120 (Pinheiro *et al.*, 2015)] was applied on positive counts only, and in the second step, a generalized linear mixed-effects model with binomial distribution [function *glmer*, in package LME4, Version 1.1-7 (Bates *et al.*, 2014)] was separately used to analyse the presence or absence of *Culicoides* in breeding sites. This approach allowed for a log transformation of the count data and implementation of the different sampling campaigns as a random effect in both models to account for seasonal variation within the data.

Data for emergence from cowpats and dungheaps were analysed separately, resulting in a total of six models with different response variables [i.e. emergence of *C. obsoletus* s.s./*C. scoticus* from dungheaps: (1a: positive counts; 1b: presence/absence), emergence of *C. chiopterus* from cowpats (2a: positive counts; 2b: presence/absence) and emergence of *C. dewulfi* from cowpats (3a: positive counts; 3b: presence/absence)]. Model selection started with the full model including the fixed effects pH, phosphor (g/kg), CN ratio, organic matter (%) and shade (three-level factor) and the random effect period (four-level factor). To the fixed effects of model 1a and 1b, the predictors trap height (m) and moisture (%) were added, and the predictor water content (%) was included in models 2a–3b. To consider potential variations caused by the different geographical regions of the farms, the longitude and latitude (decimal degrees) of the emergence traps were added as additional fixed effects to all models.

To avoid unequally sized datasets in the modelling process, observations with missing values in explanatory variables were removed prior to the analysis. Furthermore, continuous predictor variables were centred by subtracting their means from the observed scores. In order to minimize the loss of data points for the regression analysis, missing values in moisture (caused by a technical defect of the moisture probe; 52 values) and water content [cowpats of small size did not always provide enough material for both substrate samples (i.e. chemical analysis and water content), 70 values] were replaced by the mean of observations. The function *corvif* provided by Zuur *et al.* (2009) was used to calculate generalized variance inflation factors (gVIFs) in order to avoid multicollinearity among covariates. Inflation factors were calculated for the whole dataset and for subsets of the different sampling campaigns. Values of >3 led to the exclusion of the variable with the highest value and the calculation was repeated until all values were below this threshold. Consequently, potassium was excluded from the analysis as a predictor variable.

In order to identify the set of predictors with the highest explanatory power, predictor variables were sequentially dropped from the full model in a backward regression stepwise procedure by excluding the variables with the highest *P*-value and comparing every new model with the previous model using analysis of variance (ANOVA), as described by Zuur *et al.* (2009). After dropping one variable, the model was refitted with all remaining variables. These were dropped again sequentially until all remaining variables were significant. For evaluation of the final models, a conditional coefficient of determination (R^2) was calculated (Nakagawa & Schielzeth, 2013; Johnson, 2014) and residual plots were produced (histograms of Pearson's residuals, fitted values vs. Pearson's residuals). The random effect 'period' was retained in the model if it did not explain any variance [when the standard deviation (SD) was zero] in order to keep the R^2 values comparable.

In addition to the regression analysis, the individual numbers of *C. chiopterus* and *C. dewulfi* that emerged from cowpats (excluding samples with zero observations) were compared using a Wilcoxon signed-rank test and the frequency of their presence was compared using a two-sample proportions test.

Table 1. Numbers of all *Culicoides* samples (*n*) and of samples containing Obsoletus group species [*n* (positive)] from dungheaps, cowpats, compost, bark mulch in flowerbeds and water-associated substrates (water bodies) and the total numbers of individuals to emerge from these habitats, including the number of individuals non-determinable (n.d.) as a result of insufficient material conditions (e.g. missing hypopygium).

Habitat	Dungheaps	Cowpats	Compost	Bark mulch	Water bodies
n	229	225	11	23	241
n (positive)	111	73	5	2	13
♂ Obsoletus group	11 693	1 671	2	3	526
♀ Obsoletus group	12 871	1 529	7	41	245
♂ *C. obsoletus* s.s.	11 533	28	1	2	455
♂ *C. scoticus*	2	432	0	0	64
♀ *C. obsoletus* s.s./*C. scoticus*	12 852	231	7	41	245
♂ *C. chiopterus*	13	757	0	0	0
♀ *C. chiopterus*	9	795	0	0	0
♂ *C. dewulfi*	9	440	0	0	0
♀ *C. dewulfi*	0	502	0	0	0
♂ + ♀ n.d.	146	15	1	1	7
Obsoletus group					
Mean ± SD in all samples	107.3 ± 465	14.2 ± 50.3	0.8 ± 1.3	1.9 ± 6.5	3.2 ± 37.0
Mean ± SD in positive samples	221.3 ± 650	43.8 ± 80.9	1.8 ± 1.3	22.0 ± 7.1	59.3 ± 154

The mean ± standard deviation (SD) is given for the total number of Obsoletus group specimens emerging.

Results

Dungheaps

In total, 24 564 individuals of the Obsoletus group were collected in traps on dungheaps (11 693 males, 12 871 females). Of the male individuals, 11 533 were identified as *C. obsoletus* s.s. and only two as *C. scoticus*. Of the female individuals, 12 852 were classified as *C. obsoletus* s.s./*C. scoticus*. Because of the extremely low number of male *C. scoticus* specimens, it was concluded that the vast majority of these 12 852 females were *C. obsoletus* s.s. females. Small numbers of *C. chiopterus* ($n = 22$) and *C. dewulfi* ($n = 9$) specimens were also found in dungheap samples (Table 1). The average numbers of individuals to emerge from dungheaps at different farms varied widely (Table 2).

Culicoides obsoletus s.s. was present over nearly the whole range of pH, phosphor, CN ratio and moisture. Mean values for potassium in samples without Obsoletus group presence (mean ± SD 284.6 ± 36.0) exceeded the values in positive samples (22.5 ± 13.2). However, these captures were strongly affected by one extreme potassium outlier (indicated by the low median and a high mean value) (Table 3). *Culicoides obsoletus* s.s. was most abundant at pH values of 6.5–8.5, at moisture levels of about 40–60% and at trap heights up to 1.5 m (Fig. 2).

The highest numbers of individuals emerged in April and May 2013. A total of 15 116 individuals were found in 56 emergence traps (mean ± SD per trap: 269.9 ± 893.8). However, these results were strongly affected by a limited number of samples with very high numbers of individuals, whereas many samples obtained few or no *C. obsoletus* s.s. In June 2013, the total number of sampled individuals was only 5905 specimens in 58 traps (mean ± SD per trap: 101.8 ± 198.2), but overall, results were more balanced (Fig. 2). The average sex ratio (number of males divided by number of females) of *C. obsoletus* s.s. in emergence traps on dungheaps was highest during April and May 2013 and lowest in the summer months of July and August 2013 (Table 4).

Table 2. Mean ± standard deviation (SD) number of Obsoletus group specimens in emergence traps on dungheaps at the different farms (*n* = total amount of samples from the dungheap of the respective farm, including all sampling periods).

Farm	Mean ± SD	*n*
Farm 11	0	9
Farm 7	0.1 ± 0.5	14
Farm 12	0.2 ± 0.4	12
Farm 20	0.8 ± 1.5	4
Farm 8	1.8 ± 2.1	4
Farm 1	2.5 ± 6.2	10
Farm 2	4.1 ± 9.8	12
Farm 9	4.1 ± 9.9	20
Farm 4	23.8 ± 58.1	9
Farm 5	24.6 ± 47.0	13
Farm 14	44.4 ± 104.9	17
Farm 10	61.3 ± 187.1	18
Farm 16	72.0 ± 123.0	3
Farm 19	96.5 ± 270.8	17
Farm 18	113.0 ± 199.7	16
Farm 17	183.7 ± 345.0	6
Farm 3	185.4 ± 200.0	12
Farm 13	329.2 ± 486.3	14
Farm 15	605.5 ± 1466.4	18
Farm 6	No dungheap	

The regression analysis indicated that counts of *C. obsoletus* s.s. decreased with pH and trap height and increased with moisture (model 1a). Lower pH values were also associated with a higher probability of the presence of this species in dungheaps (model 1b). The model further indicated that this probability decreased along the longitudinal and latitudinal gradient (Table 5, Fig. 2). However, these effects were implemented in the model to account for geographical variation, whereas the

Table 3. Physicochemical characteristics of dungheaps and cowpats with reference to all available samples (all) and samples of substrates in which at least one specimen of the Obsoletus group was observed (positive).

Habitat	Parameter	Observations	*n*	Min	Median	Mean ± SD	Max
Dungheap	pH	All	219	5.3	7.9	7.9 ± 0.9	9.9
		Positive	110	5.4	7.8	7.7 ± 0.9	9.8
	Potassium, mg/100 g	All	216	0.1	24.8	284.6 ± 36.0	503.4
		Positive	110	0.1	19.7	22.5 ± 13.2	56.9
	Phosphor, g/kg	All	216	0.1	3.5	3.8 ± 2.2	18.5
		Positive	110	0.1	3.3	3.7 ± 2.5	18.5
	CN ratio	All	216	7.6	12.1	13.2 ± 4.0	40.0
		Positive	110	8.7	12.1	13.0 ± 4.2	40.0
	Moisture, %	All	177	0.0	37.5	34.7 ± 16.0	100
		Positive	88	0.0	39.2	35.7 ± 16.8	100
Cowpat	pH	All	221	5.3	7.3	7.2 ± 0.5	8.4
		Positive	73	5.3	6.8	7.0 ± 0.6	8.2
	Potassium, mg/100 g	All	221	0.2	5.6	6.7 ± 4.1	27.8
		Positive	73	0.2	5.7	5.4 ± 4.0	17.3
	Phosphor, g/kg	All	221	0.2	4.2	4.2 ± 2.4	13.1
		Positive	73	0.2	4.5	4.2 ± 2.4	10.7
	CN ratio	All	220	11.1	15.0	15.4 ± 2.5	23.1
		Positive	72	11.3	15.5	15.7 ± 2.5	23.1
	Water content, %	All	155	6.9	48.3	45.1 ± 20.0	85.8
		Positive	51	11.7	45.4	45.7 ± 1.1	85.8

CN ratio, carbon to nitrogen ratio; Max, maximum; Min, minimum; SD, standard deviation.

number of sampled farms along these gradients was not high enough to allow conclusions on distribution patterns. No effect of the amount of shade at sample sites was indicated by these or other regression models in the study. In model 1b, the standard variation of the random effect 'sampling period' was zero.

Cowpats

A total of 3200 individuals of the Obsoletus group emerged from cowpats, mainly comprising *C. chiopterus* (1552 specimens) and *C. dewulfi* (942 specimens). Totals of 28 male *C. obsoletus* s.s., 432 male *C. scoticus* and 231 *C. obsoletus* s.s./*C. scoticus* females were present (Table 1). *Culicoides obsoletus* s.s. occurred in four samples and *C. scoticus* in 11 samples, whereas *C. chiopterus* developed in 57 and *C. dewulfi* in 34 samples. *Culicoides chiopterus* was not observed in significantly higher numbers of individuals (Wilcoxon signed-rank test, $P = 0.86$), but was found significantly more often than *C. dewulfi* (two-sample proportions test, $P < 0.01$). Based on the numbers of samples containing either one or both of the two species, *C. dewulfi* and *C. chiopterus* occurred in 46.8% of samples. The average sex ratio of both species was highest in April–May 2013 (Table 4). The lowest sex ratio for *C. dewulfi* was observed in July–August 2013. The average sex ratio of *C. chiopterus* was low in August–September 2012 and July–August 2013.

In cowpats, Obsoletus group specimens were observed over nearly the whole range of pH, phosphor and CN ratio. Potassium values up to 27.8 mg (per 100 g) were measured but midges emerged only from samples with a maximal potassium value of 17.3 mg (Table 3). Individual numbers were generally highest in April–May 2013 (Fig. 2). A total of 1940 specimens (mean ± SD 35.3 ± 83.1) were collected from cowpats during this sampling period. According to the regression analysis, the probability of observing at least one *C. chiopterus* specimen in a cowpat (model 2b) was associated with decreasing longitude (Table 5). A lower pH level was associated with a higher abundance of *C. chiopterus* (model 2a) and a higher probability of the presence in this breeding habitat of *C. dewulfi* (model 3b). The probability of the presence of *C. dewulfi* also decreased with the longitudinal degree (model 3b), whereas the count model (3a) indicated a relationship between the abundance of this species and a lower CN ratio in cowpats. However, a low coefficient of determination indicated insufficient model fit for models 2b ($R^2 = 0.14$) and 3b ($R^2 = 0.18$). Therefore, the associated results will not be discussed further. The standard variation of the random effect 'sampling period' was zero in model 3b.

Compost, bark mulch and water-associated substrates

In five of 11 samples from compost heaps, a total of nine specimens of the Obsoletus group were found [seven females, two males (Table 1)]. One of the two males was identified as *C. obsoletus* s.s., but the second was not identifiable due to a missing hypopygium. On flowerbeds covered with bark mulch, 44 individuals of the Obsoletus group were trapped. All females were identified as *C. obsoletus* s.s./*C. scoticus*; two males were identified as *C. obsoletus* s.s. and the third male was not identifiable.

Samples from water-associated substrates contained a total of 771 specimens belonging to the Obsoletus group, all of which were categorized as *C. obsoletus* s.s./*C. scoticus*. Among male individuals, 455 were identified as *C. obsoletus* s.s. and 64 as *C. scoticus*. The 13 samples with Obsoletus group midges originated from diverse water bodies, such as ponds (five samples), ditches (four samples), slurry leakage (two samples),

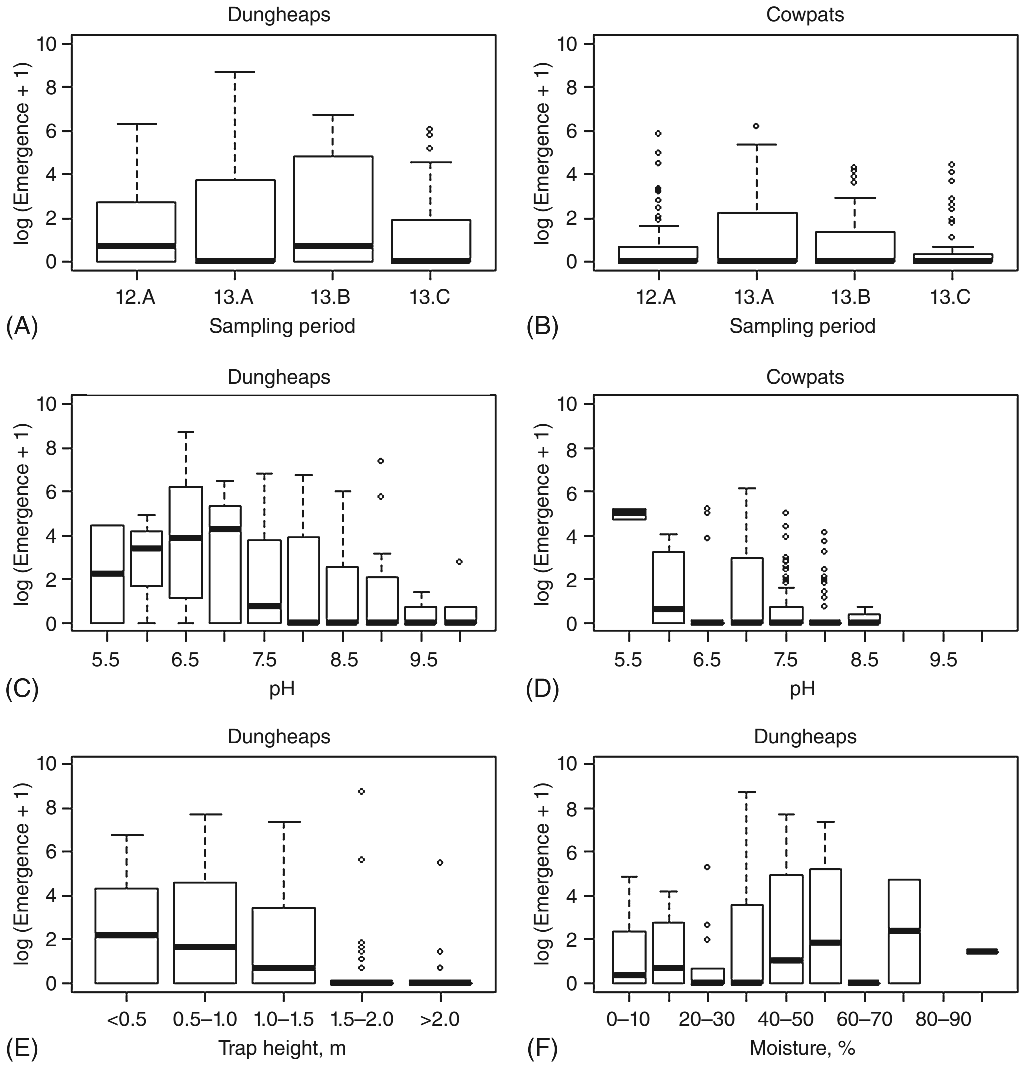

Fig. 2. Relationships between numbers of individuals of the Obsoletus group emerging from dungheaps and cowpats, and (A, B) sampling periods, (C, D) pH-values in breeding substrates (pH-values rounded to nearest 0.5), (E) trap height on dungheaps and (F) substrate moisture below traps. Numbers of individuals emerging are displayed as log + 1 to allow for better visualization. 12.A, August–September 2012; 13.A, April–May 2013; 13.B, June 2013; 13.C, July–August 2013.

rain drainage (one sample) and puddles (one sample). Comparatively low numbers of individuals (mean ± SD 1.4 ± 0.9) were collected from the majority (nine samples) of the 13 samples. However, at a pond near a dungheap, a mean ± SD of 189.5 ± 250.1 individuals emerged under four traps on mud near the water edge and on a mat of floating grass. In view of the small number of samples, no regression analysis was conducted using the data for compost, bark mulch and water-associated substrates.

Discussion

Agricultural environments offer suitable breeding substrates for the development of immature *Culicoides*. Cow dung has been mentioned earlier as an important developmental substrate for *C. chiopterus* and *C. dewulfi* (Kettle & Lawson, 1952; Campbell & Pelham-Clinton, 1960; Kremer, 1965). Single specimens of these species have also been found in pine litter, moist straw, soil polluted with chicken manure, fungi and silage residues (Laurence, 1953; Jamnback, 1965; Hackman & Meinander, 1979; Zimmer *et al.*, 2013). As the vast majority of these species emerged from cowpats, the present data emphasize a distinct preference for this type of habitat as a developmental substrate. By contrast, *C. obsoletus* s.s. developed in enormous numbers in cattle dungheaps, but was rarely present in cowpats. Although there were strong variations in the productivity of dungheaps from different farms, the emergence of several hundred individuals per trap was not uncommon. In consequence, this habitat must be considered as one of the most important sources of *C. obsoletus* s.s. in rural areas.

Table 4. Sex ratio (number of males divided by number of females) of *Culicoides* that emerged from dungheaps and cowpats over the four sampling periods of August–September 2012 (12.A), April–May 2013 (13.A), June 2013 (13.B) and July–August 2013 (13.C).

		Sampling period				
Substrate	Species	All periods Mean ± SD	12.A Mean ± SD	13.A Mean ± SD	13.B Mean ± SD	13.C Mean ± SD
Dungheap	*C. obsoletus* s.s.	0.6 ± 1.1	0.2 ± 0.2	1.5 ± 1.8	0.9 ± 1.0	0.1 ± 0.1
Cowpat	*C. chiopterus*	0.9 ± 1.2	0.4 ± 0.6	1.4 ± 1.1	1.2 ± 1.7	0.4 ± 0.6
Cowpat	*C. dewulfi*	1.2 ± 2.4	0.6 ± 0.9	3 ± 3.8	0.6 ± 0.5	0.2 ± 0.2

SD, standard deviation.

Table 5. Regression coefficients estimates, standard errors (SE) and *P*-values for fixed effects retained in the positive count models (*lme*) and binomial models (*glmer*, presence vs. absence) and standard deviation (SD) of the random effect ‘period’.

Response/model coefficients	Estimate		SE	SD	*P*-value	*n*	AIC	R^2
Emergence of *Culicoides obsoletus* s.s. from dungheaps: positive counts						105	446.3	0.27
pH	−0.94	±	0.21		<0.001			
Moisture	0.04	±	0.01		<0.01			
Period (random effect)				0.51				
Emergence of *C. obsoletus* s.s. from dungheaps: presence vs. absence						210	239.8	0.43
pH	−0.69	±	0.21		<0.001			
Trap height	−1.36	±	0.22		<0.001			
Longitude	−0.26	±	0.1		<0.01			
Latitude	−0.22	±	0.09		<0.05			
Period (random effect)				0				
Emergence of *Culicoides chiopterus* from cowpats: positive counts						50	171.9	0.34
pH	−1.36	±	0.30		<0.001			
Period (random effect)				0.0004				
Emergence of *C. chiopterus* from cowpats: presence vs. absence						192	210.6	0.14
Longitude	−0.36	±	0.10		<0.001			
Period (random effect)				0.20				
Emergence of *Culicoides dewulfi* from cowpats: positive counts						27	102.6	0.22
CN ratio	0.29	±	0.10		<0.05			
Period (random effect)				0.0004				
Emergence of *C. dewulfi* from cowpats: presence vs. absence						193	149.9	0.18
pH	−0.88	±	0.40		<0.01			
Longitude	−0.34	±	0.13		<0.05			
Period (random effect)				0				

n, number of observations analysed; AIC, Akaike information criterion; R^2, conditional coefficient of determination) for final variable sets are given; CN ratio, carbon to nitrogen ratio.

No *C. dewulfi* or *C. chiopterus* specimens were collected from semi-aquatic substrates, bark mulch or compost, whereas they were occasionally found in emergence traps on dungheaps. Accordingly, single specimens of *C. dewulfi* emerged from old composted manure in Spain (González *et al.*, 2013). Similar results were reported in a recent survey in Belgium (Zimmer *et al.*, 2014), where small numbers of *C. dewulfi* or *C. chiopterus* were found in various substrates (e.g. the soil of a livestock trampling area, components of a chicken coop, food residues and molehill soil), but the majority of individuals also emerged from cowpats.

There were clear species-specific preferences of *C. dewulfi* and *C. chiopterus* for cowpats, and of *C. obsoletus* s.s. for dungheaps. By contrast with cowpats lying outside on pastures, the material of dungheaps generally includes litter from inside cattle stables. Thus, the results may indicate endophilic behaviour in *C. obsoletus* s.s. Accordingly, the species has been found to enter buildings and to emerge from dung inside stables in previous studies (Baldet *et al.*, 2008; Meiswinkel *et al.*, 2008b; Zimmer *et al.*, 2010; Ninio *et al.*, 2011b). Possibly, *C. obsoletus* s.s. prefers to feed on hosts indoors. Furthermore, the possibility that oviposition takes places inside stables and that the colonised material is later added to dungheaps outside, from which midges emerge, cannot be excluded. The differences in substrate composition between dungheaps and cowpats may include discrepancies in the food sources available to immature stages of the different species. Overall, the two habitat types also differed in their abiotic character (e.g. CN ratios and pH values were distributed over a wider range in dungheaps, reflecting the general variability of this habitat). Generally, pH values were slightly higher in dungheaps than in cowpats (Fig. 2, Table 4).

The abundance of *Culicoides* is often reflected by the physicochemical characteristics of its habitat (Purse *et al.*, 2015). In the present study, the pH value of the substrates appeared to be related to the presence and abundance of *Culicoides*. The highest numbers of Obsoletus group individuals emerged from cowpats with pH values of 5.5–7.0, whereas in dungheaps the largest numbers of specimens to emerge were associated with pH values of 6.5–8.0. For all three species, *C. obsoletus* s.s., *C. chiopterus* and *C. dewulfi*, a decrease in pH was related to either a higher probability of presence or a higher abundance at a breeding site or both, according to the regression analysis. The abundance of *C. obsoletus* s.s. in dungheaps was further related to increasing substrate moisture. High individual numbers were associated with moisture levels of around 40–60%, whereas the average moisture in dungheaps was around 30%. A positive impact of substrate moisture and pH on the presence of this species was reported in a study from England (Harrup *et al.*, 2013). By contrast, no relationship between the water content in cowpats and the emergence of *C. dewulfi* or *C. chiopterus* was indicated by the regression analysis.

Development in cowpats may imply the need for a physiological tolerance of drought to some extent. Kettle & Lawson (1952) assumed older cowpats to be among the driest habitats of *Culicoides*. In the present study, *C. dewulfi* and *C. chiopterus* successfully emerged from cowpats with water content of < 20%. However, it should be noted that the water content may have been higher at the beginning of the 14-day sampling period, and that other chemical parameters in the investigated habitats may have changed over time. For *Culicoides brevitarsis* Kieffer, Bishop *et al.* (1996) documented that a vertical movement of immature stages within cowpats was associated with moisture content. The present study did not allow for an assessment of moisture differences within cowpats. Thus, it is possible that *C. dewulfi* and *C. chiopterus* prefer higher moisture levels within the substrate, but that their development success may not be noticeably affected by an overall decline in water content. Moreover, if a colonized cowpat dries out, the larvae would probably not have the option of leaving the habitat, whereas larvae in a dungheap may be able to migrate to more favourable positions within the heap (e.g. into substrates with higher moisture levels).

Leaving aside considerations of water content, the age of the cowpat may play an important role in oviposition and successful development. In the present study, attempts were made to select cowpats that were at least a few days old, but aged material was also investigated. Without knowledge of the time of cowpat deposition, it is not possible to make a reliable judgement of cowpat age. However, *Culicoides* were found to emerge from cowpats that appeared to be at least a week old at the beginning of the sampling period, as well as from apparently older pats, of which some had been penetrated by plants or were even partly decomposed. Campbell & Kettle (1976) assumed increasing oviposition rates of *C. brevitarsis* on cowpats up to the age of 7 days in Australia. These authors suggested that, after that time, the crust may become too thick to allow oviposition or development of eggs. It is possible that, in temperate regions, the crust does not thicken as fast and thus affords a longer period for successful oviposition.

In addition to moisture and pH, the presence of *C. obsoletus* s.s. was also related to the position of the emergence trap on the dungheap. The highest numbers of individuals were found at heights of 0–1.5 m and the probability of observing the species decreased with trap height. This result may reflect female oviposition at low to moderate heights. According to the literature, *C. obsoletus* s.s. is most common at ground level (Kettle, 1962). Service (1971) sampled the highest numbers of *C. obsoletus* s.s. with suction traps at a height of 23 cm (comparing heights between 23 cm and 550 cm), but these numbers represented primarily unfed females. González *et al.* (2013) found the largest numbers of *C. obsoletus* s.s. in old composted sheep dung. Thus, a relationship between the presence of *C. obsoletus* s.s. and the age of the dung may be conceivable, given that fresh dung is regularly added to the tops of heaps by farmers, and thus the results of higher traps derive from fresher dung.

Culicoides obsoletus s.s. was the most abundant species in the present survey. Not only is its occupation of dungheaps as a breeding habitat highly successful, but it was also the only species to be identified in samples from all of the habitat types investigated (i.e. cowpats, bark mulch, compost and water-associated substrates), although it was not generally as abundant in these habitats. Its presence in cowpats was recorded in only four of 225 samples and, with the exception of those emerging from a pond polluted by dungheap residues, only a few *C. obsoletus* s.s. emerged from a limited number of water-associated habitats. The species of the Obsoletus group seem to avoid substrates that are likely to be inundated. Accordingly, Nevill *et al.* (2007) postulated that pupae of the subgenus *Avaritia* drown when submerged and thus these species are restricted to moist, but not waterlogged, substrates.

Prior to the current findings, *C. obsoletus* s.s. has been known to breed in a variety of habitats, as summarized in several previously published studies (e.g. González *et al.*, 2013; Harrup *et al.*, 2013; Zimmer *et al.*, 2014). By contrast, information on the breeding sites of *C. scoticus* is limited. Although this species is commonly found in light trap catches (e.g. Sarto i Monteys & Saiz-Ardanaz, 2003; Baldet *et al.*, 2004; Balczun *et al.*, 2009; Nielsen *et al.*, 2010), most studies report only low numbers of larvae or emerged individuals [e.g. in the mud of a wheel track in woodland, different species of fungi, composted manure or silage residues (Buxton, 1960; Kremer, 1965; Hackman & Meinander, 1979; González *et al.*, 2013; Zimmer *et al.*, 2013)]. A larger number of *C. scoticus* (584 individuals) was found to develop in decaying leaves in association with the parasitic flower plant *Lathraea clandestina* L. (González *et al.*, 2013). In addition, in the present survey, only a small number of breeding sites produced considerable numbers of *C. scoticus* specimens, which were mostly associated with *C. obsoletus* s.s. One habitat was the mud around a small pond, interspersed with decaying oak leaves and residues from a former dungheap nearby. A total of 64 *C. scoticus* males were found in three samples from this habitat. However, the largest numbers of *C. scoticus* were found in four cowpats from another farm in April–May 2013 (413 *C. scoticus* males). Given the diversity of these records, it is apparent that the typical breeding habitat of *C. scoticus* cannot yet be delimited.

Overall, very high numbers of individuals emerged from cowpats and dungheaps in April–May 2013. It is most likely that these represent the overwintering population as *Culicoides* of the Obsoletus group overwinter as larvae in these substrates (Lühken *et al.*, 2014b; Steinke *et al.*, 2015). The average sex ratios of *C. obsoletus* s.s. emerging from dungheaps and of *C. chiopterus* and *C. dewulfi* emerging from cowpats were also highest during that sampling period and decreased in summer. The data collected in April 2013 most likely covered the onset of emergence in that year. Under this assumption, the biased sex ratio may well be the result of protandry [i.e. the earlier emergence of males, which is a common aspect of insect phenology (Morbey & Ydenberg, 2001) and has also been observed for *C. impunctatus* (Boorman & Goddard, 1970; Blackwell *et al.*, 1992)].

Large numbers of individuals of *C. obsoletus* s.s. were also sampled in June 2013. Further emergence peaks may have remained undetected with the present sampling design. Nevertheless, it is suggested that control measures concentrate on this first population peak following winter as its successful reduction may have an impact on subsequent populations.

As the present study shows, large numbers of *C. obsoletus* s.s. develop in dungheaps and hence this habitat would appear to represent a good starting point for potential measures in rural surroundings. A previous approach in this context involved covering dungheaps with tarpaulins (Harrup *et al.*, 2014). However, simultaneous light trap catches revealed no differences in *Culicoides* populations in comparison with untreated farms. The authors discuss the potential emergence from other local habitats as a possible reason for this. Indeed, the success of control measures is most likely to depend on local conditions. As already mentioned, *Culicoides* densities in dungheaps varied strongly between farms in the present study, and therefore control measures may not be equally effective at all sites. Thus, dungheaps should still be considered as a potential target for control measures in the future because, in this habitat, high densities of immature stages can be spatially confined in relatively small areas. In order to suppress adult *Culicoides* populations, control measures should aim at the larval stage, ideally prior to the emergence of the overwintering individuals in spring. However, experimental studies will be necessary to investigate the practical potential of the disturbance of immature development in dungheaps, especially with regard to the compatibility with agricultural activities.

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11.1.4 MANUSCRIPT IV

Impact of freezing on the emergence of *Culicoides chiopterus* and *Culicoides dewulfi* from bovine dung

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Short Communication

Impact of freezing on the emergence of *Culicoides chiopterus* and *Culicoides dewulfi* from bovine dung

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ABSTRACT

The emergence of *Culicoides chiopterus* (Meigen), 1830 and *C. dewulfi* Goetghebuer, 1936 (Diptera: Ceratopogonidae) from cowpats in northwestern Germany was investigated. In order to investigate the survival of both species at low temperatures, cowpat subsamples were frozen for 48 h at −18 and −21 °C. Emergence from frozen and non-frozen samples was compared. The number of emerging adults of *C. chiopterus* from samples frozen at −18 °C was greatly reduced and no emergence was observed from samples frozen at −21 °C. No adult *C. dewulfi* emerged from frozen samples, suggesting this species is less resistant to these temperatures, compared to *C. chiopterus*.

1. Introduction

Several species of the genus *Culicoides* (Diptera: Ceratopogonidae) are known to transmit pathogens that infect cattle and other livestock (Mellor et al., 2000). Members of the Obsoletus group belong to the main vectors for the bluetongue virus (BTV) and Schmallenberg virus (SBV) in central and northern Europe. For BTV, *Culicoides obsoletus* sensu stricto has been identified as a main vector, but also *C. chiopterus* and *C. dewulfi* are among the potential vectors for this virus (Carpenter et al., 2009 (review); Hoffmann et al., 2009). Furthermore, there is evidence that they are potentially involved in the transmission of SBV (De Regge et al., 2012; Elbers et al., 2013).

While the veterinary importance of the genus is beyond question, knowledge of the ecology of *Culicoides*, e.g., on overwintering mechanisms, is still fragmentary. Many species in temperate regions overwinter in the larval stage (Becker, 1960; Jones, 1967; Szadziewski et al., 1997). Concluding from the emergence of *C. obsoletus* in the spring, dung heaps are assumed to be habitats for overwintering individuals of this species in the United Kingdom (Harrup et al., 2013). Larvae of *C. chiopterus* and *C. dewulfi* are known to develop in cowpats (Kettle and Lawson, 1952), and can be found overwintering in this habitat on cattle pastures (Steinke et al., 2014).

The present study focused on the survival of *C. chiopterus* and *C. dewulfi* at very low temperatures to investigate the potential impact of severe winters on the population. For this purpose, overwintering larvae within cowpats were frozen in the laboratory to investigate subsequent emergence. A preliminary study demonstrated that larvae of *C. chiopterus* can survive freezing at −15 °C (data not shown). Thus, we investigated emergence after freezing at −18 °C and −21 °C to determine the temperature level precluding survival.

2. Methods

Sampling was performed on a pasture (GPS coordinates: N53 9.985 E8 8.771) belonging to an organic cattle farm in northwestern Germany in January 2014. Pasturing

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was stopped in the preceding autumn. Thus, cowpats used for the experiments had a minimum age of 3–4 months. Several days prior to the main study, small subsamples (approximately 10 g each) were collected from the edges of 15 cowpats. *Culicoides* larvae were extracted from the samples with an adapted Berlese method (Steinke et al., 2014) to ensure that only colonised cowpats were used in the experiment. In 12 cowpats, *Culicoides* larvae were found and 10 of these “positive” cowpats were randomly selected for further use. These cowpats were removed whole along with the soil layer (down to 3–4 cm) underneath and transported to the laboratory. Each pat was divided into three triangular pieces of approximately equal size. One subsample of each cowpat was frozen for 48 h at −18 °C and another one at −21 °C in two separate deep freezers. The third subsample served as the control and was stored outdoors. During freezing, the temperature within the cowpats was monitored with miniature data loggers (iButton DS1921G, Maxim Integrated) that were carefully inserted into the centre of the subsamples. Additionally, the temperature in the deep freezers was monitored (HOBO Pendant® Temperature/Alarm Data Logger 8 K, ONSET). The time between sampling and the initiation of freezing was approximately 1 h. After 48 h, all samples were placed in emergence traps in a greenhouse under natural daylight. Rearing temperature (Hobo Pro v2, ONSET [in combination with solar radiation shield RS3]/Resolution: 0.02 °C at 25 °C, Bourne, MA, USA) was 16.0 ± 2.9 °C with a relative air humidity of 68.1 ± 12.6%. The traps were composed of plastic buckets with two lateral aeration windows and a transparent top collection container. Emerging insects fly into the top container and are preserved in salt solution. Trapped insects were removed every second or third day and cowpat samples were moistened with tap water. *Culicoides* biting midges were sorted out after emergence and identified to the species level following the key of Campbell and Pelham-Clinton (1960).

The temperature within cowpats was monitored in the same cattle pasture for 2 months prior to the experiment (20/11/13–23/02/14) in order to determine the temperature range overwintering larvae were exposed to naturally in advance of the freezing treatment. Three cowpats of similar size and height were chosen (diameter: 30 ± 4 cm, height: 4 ± 1 cm). The temperature was again measured with miniature data loggers that were inserted in the top half and the bottom half of each pat. Air temperature was measured at the edge of the pasture (10–20 m from the cowpats) at a height of 1 m (Hobo Pro v2, ONSET [in combination with solar radiation shield RS3]/Resolution: 0.02 °C at 25 °C, Bourne, MA, USA). The temperature of the air and cowpats was measured hourly. All statistical analyses were carried out with the program R (R Development Core Team, 2014). A confidence level of 5% was used to define statistically significant differences. Numbers of emerged individuals from different treatments were compared with the Wilcoxon signed-rank test.

3. Results

Minimal temperatures within cowpat subsamples (−18.2 ± 0.9 °C [mean ± SD]) frozen at −18 °C were reached towards the end of the 48 h period (46.3 ± 1.9 h). Subsamples frozen at −21 °C reached a minimal temperature of −23.4 ± 2.4 °C after 42.7 ± 4.8 h. From samples frozen at −21 °C, no *Culicoides* emerged, whereas a total of 33 *C. chiopterus* (20 females/12 males) emerged from 3 of the 10 subsamples frozen at −18 °C (Table 1). A total of 1361 *Culicoides* midges eclosed from the control samples with a high variation of individual numbers among the cowpats (62.1 ± 55.5 per subsample). Of these individuals, 621 (45.6%) were identified as *C. chiopterus* (331 females/290 males) and 711 (52.2%) individuals as *C. dewulfi* (148 females/563 males). In comparison with the control samples, the number of *C. chiopterus* individuals that emerged from subsamples frozen at −18 °C was significantly lower (Wilcoxon signed-rank test, $n = 10$, $p < 0.001$).

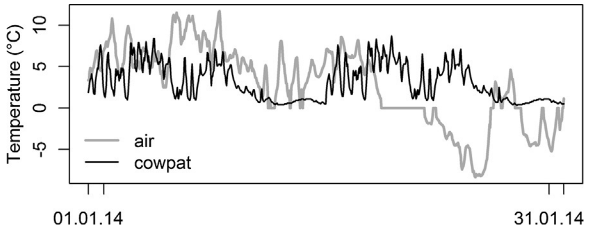

Fig. 1. Hourly measured air temperature (°C) (grey thick line) on a pasture in northwestern Germany (measured at a height of 1 m) in comparison to the average temperature measured within cowpats on the pasture (black thin line) from 1st to 31st January 2014 (temperature was measured within three cowpats, in the top half and the bottom half of each sample).

According to the temperature monitoring on the pasture, it is unlikely that the overwintering larvae were confronted with freezing in advance of the main experiment. When the air temperature fell below 0 °C, cowpat temperatures did not follow but remained close to the freezing point (Fig. 1). The average temperature measured within cowpats in the period from 20/11/13 to 23/02/14 was 3.5 ± 2.3 °C.

4. Discussion

Larvae of *C. variipennis*, the primary vector of the bluetongue virus in North America (reviewed by Tabachnick, 1996), are able to survive freezing at a temperature of −2.2 °C over a period of 6 weeks (62% of exposed larvae; Rowley, 1967). Icebound larvae of the same species collected from frozen pond mud were alive and active after thawing (Vaughan and Turner, 1987). The present study demonstrates the competence of larvae of another veterinary important species, *C. chiopterus*, to survive temperatures far below the freezing point. The lowest temperature in the subsamples from which emergence was observed was −18 °C, while no emergence could be observed after freezing at −21 °C. The lower threshold for survival of *C. dewulfi* was assumed to be at a higher temperature, as this species was abundant in the control samples, but did not emerge from any samples after freezing, indicating a lower physical resistance towards the low temperatures tested in the present study. *Culicoides* are generally able to survive in severe climates.

Table 1
Emergence of *Culicoides chiopterus* and *Culicoides dewulfi* from subsamples of cowpats frozen at −18 °C for 48 h and non-frozen samples (control). No emergence was observed from subsamples frozen at −21 °C. Temperatures given are minimal temperatures reached within subsamples during the freezing treatment.

Sample	−18 °C		−21 °C		Control	
	C. chiopterus	°C (min)	*C. chiopterus/C. dewulfi*	°C (min)	*C. chiopterus*	*C. dewulfi*
C1	0	−19.4	0	−26.2	81	38
C2	29	−17.1	0	−20.9	66	37
C3	0	−19	0	−22	182	177
C5	0	−17	0	−22.4	53	171
C6	0	−17.1	0	−21.1	17	9
C8	1	−17.6	0	−20.6	41	52
C9	3	−18.4	0	−25.8	22	45
C12	0	−18	0	−25.8	141	141
C13	0	−19.1	0	−25.8	10	35
C15	0	−19	0	NA	8	6
Sum of individuals/mean ± SD (°C)	33	−18.2 ± 0.9	0	−23.4 ± 2.4	621	711

Many species have been recorded in Siberia, including *C. chiopterus*. In contrast to *C. dewulfi*, this species has been reported from various regions in Russia, including Western Siberia (reviewed by Sprygin et al., 2014). Possibly, the potential differences in physiological resistance to freezing could be interpreted as a reflection of this distributional divergence.

Only a few insect species can survive actual freezing of the body, but there are many examples of taxa that are able to decrease the temperature point at which their bodily fluids freeze to below 0 °C (reviewed by Lee et al., 1993). It is not clear what mechanisms allow *Culicoides* species to survive exposure to sub-zero temperatures. Vaughan and Turner (1987) reported elevated glycerol levels in winter larvae of *C. variipennis* compared to individuals sampled in the summer; glycerol is among the most common cryoprotectants in the overwintering mechanisms of insects (reviewed by Lee et al., 1993). Further, Nunamaker (1993) indicated that adult *C. variipennis sonorensis* can survive exposure to a temperature of −10 °C for much longer after prior acclimatisation at 5 °C for 1 h, indicating rapid physiological adaptation to low temperatures.

The temperature of the upper soil layer does not directly follow air temperature below 0 °C, but remains near the freezing point until the ground surface is completely frozen (Beltrami, 2001). Accordingly, the temperature monitored within cowpats during the present study remained close to the freezing point when the air temperature fell below 0 °C (Fig. 1). At the same time, larvae of *C. chiopterus*, overwintering in cowpats, can survive temperatures down to −18 °C. In Germany, topsoil temperatures in this range are possible, but rather uncommon (National Meteorological Service of Germany, 2014a,b). Thus, a significant reduction in the overwintering population of *C. chiopterus*, caused by low temperatures during winter, seems unlikely. In any event, further research will be necessary to determine the exact temperature point and duration of freezing that are likely to have an impact on the *Culicoides* population. More detailed information could allow for a better assessment of the potential survival of the overwintering population during severe winters.

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11.1.5 MANUSCRIPT V

Impact of experimental flooding on larvae and pupae of dung-breeding *Culicoides* (Diptera: Ceratopogonidae)

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Impact of flooding on immature *Culicoides*

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Key words

cowpat, development, floating, overwintering, swimming, vector

Abstract

Culicoides biting midges spend the greatest part of their life in the larval stage. However, the knowledge on the immature stages and the impact of abiotic factors on their development is still poor. Therefore, we investigated the effect of flooding on the larvae and pupae of *Culicoides chiopterus* (Meigen) and *C. dewulfi* Goetghebuer. In water, the larvae of both species showed specific motion sequences, head-to-tail flexions and sinuous flexions, at slow rates, but were not able to swim. Flooding of larvae for 24 hours did not affect the number of emerging adults; flooding of pupae significantly reduced the emergence rate of *C. chiopterus* by 23%, compared to the control group, while *C. dewulfi* was not significantly affected. Pupae were not able to float and no pupae survived flooding for ten days. After flooding of larvae for ten days, 50% of *C. chiopterus* and 4% of *C. dewulfi* completed the pre-adult development. During this treatment, 84% of *C. chiopterus* and 48% of *C. dewulfi* larvae pupated in water. Using larvae of a summer population, in both species about 60% showed this behaviour, which significantly reduced the development success in both species. After pupation in water, 4% of *C. chiopterus* and 1% of *C. dewulfi* developed to adult midges (8% and 6%, respectively, of larvae that did pupate in water). The behaviour in water and the negative impact of long-term flooding on the development reflects the adaption to the development in a terrestrial habitat.

Introduction

The immature stages of *Culicoides* develop in a variety of breeding habitats, e.g., streams, bogland, salt-marsh, tree-holes, leaf litter, fungi or animal dung (Kettle and Lawson, 1952; Murray, 1957; Buxton, 1960; Schmidtmann; 2006). The hydrological conditions of these breeding sites differ strongly and can change rapidly. Some species prefer to develop in waterlogged habitats, as *C. nubeculosus* (Meigen) and *C. circumscriptus* Kieffer (Downes, 1950; Foxi and Delrio, 2010). Other species seem to avoid inundated substrates, as those of the subgenus *Avaritia* (Nevill et al., 2007). This taxa includes some of the relevant vectors of veterinary important pathogens, i.e., *C. imicola* Kieffer and the species of the Obsoletus Group, that are involved in the transmission of the African horse sickness virus and the bluetongue virus (reviewed by Meiswinkel et al., 2004 and Carpenter et al., 2009; Hoffmann et al., 2009). *C. imicola* Kieffer is common in moist and muddy habitats, usually lacking surface-water (Braverman and Galun, 1974; Braverman, 1978; Mellor and Pitzolis, 1979; Foxi and Delrio, 2010). Instead, *C. chiopterus* and *C. dewulfi* develop in cowpats, which are among the driest breeding habitats colonised by *Culicoides* (Kettle and Lawson, 1952).

Many species possess behavioural or physiologic adaptations for environmental changes in the respective habitat, e.g., for flooding. While the larvae of *C. nubeculosus*, *C. circumscriptus* or *C. variipennis* are good swimmers (Megahed, 1956; Becker, 1960; Linley, 1986), *C. brevitarsis* or *C. imicola* lack this ability (Cannon and Rye, 1966; Bellis et al., 2014). Pupae of *Culicoides* are classified into four groups according to the behaviour in water (Dyce and Murray, 1966; Nevill, 1967). In group 1, pupae can float to the surface but are unable to submerge. The species of group 2 are capable of both, i.e., floating and sinking back under water. Pupae of the third group are unable to float and will burrow into the substrate when flooded. In the fourth group, pupae of *C. imicola* do not float but remain on the ground and die within a few days.

Larvae of the salt-marsh species *C. furens* (Poey) survive immersion for up to 14 days (Magnon et al., 1990). However, in terrestrial species, the resistance to flooding has rarely been investigated. In the dung-breeding species *C. chiopterus* and *C. dewulfi* flooding of the breeding habitat prevents an emergence of adults (Lühken et al., 2014). However, it remained unclear if the results were related to a sensitivity of larvae or pupae. A different susceptibility of both stages is known for other species of Diptera and other abiotic factors, e.g., a higher or lower resistance of pupae than larvae to freezing (e.g., Mugnano et al., 1996; Jensen et al., 2007). Therefore, the present study investigates the behaviour of larvae in water and the impact of short and long term flooding on the larvae and pupae of these two species separately.

Methods

Sampling of larvae of *Culicoides*

Culicoides larvae originated from cowpats, sampled on a pasture belonging to an organic cattle farm in northwestern Germany (GPS coordinates: N53 9.985, E8 8.771). Larvae were extracted using the Berlese method (see Steinke et al., 2014 for methodical details), collected in tap water and morphologically identified to species level according to the key of Kettle and Lawson (1952). In September and October 2015, larvae were sampled from 20 different cowpats for the recording of the motion sequences of larvae in water. The sampling for the flooding experiments (20 cowpats) took place in February and March 2015. In order to determine the pupation rate in water, further larvae were extracted from a total of 45 cowpats in July and August 2015.

Recording of the motion sequences of larvae in water

After the transfer of larvae into water, these were filmed for at least 15 minutes per specimen, using a webcam (Microsoft LifeCam VX-3000), in combination with a stereomicroscope at low magnification (10x). The motions of 21 larvae at room temperature were analysed and the rate of two different types of larval body motions determined. In the head-to-tail flexion the larval body was strongly bent, head and tail approaching each other and sometimes touching;

then head and tail slowly weaved to the other side until they were close again. The head-to-tail flexions were rarely performed at regular intervals, and the rate was calculated on the basis of several single flexions, i.e., a single weave at one side from the head to the tail. The second type was a sinuous flexion which resembled a snake like motion (see figures and detailed description of swimming *C. variipennis* provided by Linley (1986). Linley (1986) defined a complete flexion as the weave to one side and back, returning the head to the initial position. This definition was adopted for the present study, and the sinuous flexion rate was calculated as the number of complete flexions per second.

After the records, larvae were reared to adults in order to determine the gender and verify the identification of larvae by identifying the adults according to the key of Campbell and Pelham-Clinton (1960). Therefore, larvae were kept in glass petri dishes (60x15mm) which were filled to one third with moistened sterile silica sand (Rosnerski, Königslutter, Germany) on a bottom layer of paper tissue. In a preliminary study, the sand was a sufficient maintenance substrate for fourth instar larvae of *C. chiopterus.* and *C. dewulfi*.

Flooding and maintenance of larvae

The dishes described in the previous section were also used for the flooding experiments, because the sand does not disperse if the dishes are flooded and facilitates the detection of immature stages and emerged adults. Groups of ten larvae per petri dish were transferred. After 24 hours (time for larvae to dig into the substrate) a total of 100 larvae of *C. chiopterus* and 100 larvae of *C. dewulfi* were flooded for 24 hours and the identical number for ten days. Ten other groups, the controls, were not flooded. For flooding, tap water was carefully added into the petri dishes, until two thirds were filled up. After the period of exposure, the water was carefully withdrawn with a pipette under visual control using a stereomicroscope. The dishes were maintained at room temperature (20.4 ± 1.1 °C) and natural daylight. After emergence of adult *Culicoides*, the dishes were placed in a small gauze bag and opened until all adults had left the dish, attracted by the light of a window nearby. This procedure was continued until no further adults emerged for at least 14 subsequent days. Generally, no pupae and larvae were detected afterwards. The collected adults were used to verify larval identifications (see above). The effect of the different treatments was statistically evaluated by comparing the number of adults in control and flooded groups, using the two-sample proportions test of the programme R, version 3.1.2 (R Core Team, 2014).

Flooding and maintenance of pupae

Larvae of *C. chiopterus* and *C. dewulfi* were maintained in the petri dishes and controlled daily for pupations. After recording the location of pupae (lying on the sand or under the paper tissue or embedded into the sand), they were carefully transferred within the surrounding substrate into the substrate of new dishes, using a micro-spatula. Pupae that lay loosely on the sand were transferred in the same position. If the cuticula of a fresh pupa had not hardened, the pupa was transferred on the next day. Directly after the transfer, ten groups, each composing of ten pupae of each species, were flooded for periods of 24 hours or ten days. In five dishes, the control groups, 50 pupae of each species, were transferred to new dishes but not flooded. After removal of the water from the flooded groups, all dishes were controlled daily for emerged adults, which were removed daily for the determination of species and gender (see above).

Maintenance of larvae for records of pupation in water

After flooding of larvae for ten days exposures, many pupated in water. Since these larvae originated from cowpats collected during winter, a further experiment was conducted to clarify, if summer larvae will also pupate when flooded for a longer period of time, and whether they are able to interrupt the development under such unfavourable conditions. For this purpose, 45 larvae of *C. chiopterus* and 68 larvae of *C. dewulfi* were transferred as small groups (2 - 20 larvae) into petri dishes filled with tap water (but no sand) and kept at room temperature (23.4 ± 1.4 °C) and natural daylight. After pupation in water, pupae were directly transferred to new dishes containing tap water (1 pupa per dish). Ten days after the transfer of the larvae into water, all pupae and larvae were transferred to single dishes containing sand. Emerged adults were removed daily, and the gender and species of adults was determined (see above). Proportion tests were used to compare the development success of individuals that pupated in water to the number of individuals that did not show this behaviour. Moreover, the number of pupated individuals in water of summer and winter populations was compared.

Results

Motion sequences of larvae in water

The larvae used in these experiments developed to six males and eight females of *C. chiopterus* and one male and one female of *C. dewulfi*, whereas another five individuals were identified as *C. obsoletus* sensu stricto (s.s.) (Meigen) or *C. scoticus* Downes and Kettle (all females). In no species did head-to-tail flexions result in swimming forward or backward. These flexions (single weaves) occurred at a rate of 0.8 ± 0.2 flexions/second (f/s, n = 14) in *C. chiopterus* and 0.7 ± 0.1 f/s (n = 2) in *C. dewulfi* larvae. The sinuous flexions did, at the most, cause minimal forward locomotion in water. In *C. chiopterus* a rate of 1.6 ± 0.4 f/s (n = 7) and in *C. dewulfi*, a rate of 1.3 f/s (n = 1) was determined. In larvae of *C. obsoletus* s.s./ *C. scoticus*, this rate was about twice as fast (3.0 ± 0.4 f/s, n = 5). Beside these defined motions, the *Culicoides* larvae often showed other irregular or interrupted movements or remained motionless for up to several minutes.

Behaviour and survival of flooded larvae

After the transfer of larvae into the dishes, some burrowed immediately into the substrate. However, most individuals crawled around on the substrate for several hours. Within 24 hours, most larvae had burrowed into the substrate. A total of 93 *C. chiopterus* developed to adults in controls, 91 after flooding for 24 hours and 50 individuals after flooding for 10 days, in the latter group significantly less compared to controls ($p < 0.001$) (Table 1). Less adults of *C. dewulfi* developed successfully under the same conditions, i.e., 78 out of 100 control larvae. In the group of larvae flooded for 24 hours, 87 adults emerged, but significantly less, four, after flooding for a period of ten days ($p < 0.001$ in two-sample proportion test). Among the larvae, flooded for ten days, 48 *C. chiopterus* and 84 *C. dewulfi* pupated in water. All species identifications of emerged adults confirmed the identification of larvae.

Behaviour and survival of flooded pupae

Some pupae were found lying on the sand (23.3% *C. chiopterus*, 23.8% *C. dewulfi*) or under the paper tissue at the bottom of the dish (19.3% *C. chiopterus*, 13.2% *C. dewulfi*). However, the majority (56.4% *C. chiopterus*, 60.3% *C. dewulfi*) pupated in narrow burrows in the sand with the head or cephalothorax protruding. Sometimes, larvae with dilated thoracic segments were observed sitting in such burrows.

During flooding, no floating of pupae was observed. Burrowed pupae digged themselves out the substrate and lay on the sand, moving only occasionally. Ninety-six and 90% out of 50 control pupae of *C. chiopterus* and *C. dewulfi*, respectively, developed to adults (Table 1). Out of the 100 pupae flooded for 24 hours, 77 adults of *C. chiopterus* and 90 of *C. dewulfi* emerged, in the latter statistically significantly less than in controls ($p < 0.001$ in two-sample proportion test). No pupae survived flooding for a period of ten days. In the control groups (transfer to new petri dish without flooding, 50 individuals of each species) nearly all individuals successfully developed to adult *Culicoides*, i.e., 48 *C. chiopterus* and 45 *C. dewulfi* and (Table 1).

Rate of pupation of larvae in water

Of the larvae obtained from cowpats during summer and maintained in tap water for ten days, 26 *C. chiopterus* (57.8%) and 41 *C. dewulfi* (60.3%) pupated in water. Of the specimens that had pupated in water, four *C. chiopterus* (15.4%) and one *C. dewulfi* (2.4%) successfully finished the development after removal from the water. Another three *C. dewulfi* died during the process of eclosion. In the groups of larvae that did not pupate in water, significantly more developed to adult midges in comparison to those that did pupate in water, i.e., eight out of 19 *C. chiopterus* (42.1%) and six out of 27 *C. dewulfi* (22.2%, $p < 0.05$ in two-sample proportion test). Of larvae extracted in summer, significantly less *C. dewulfi* pupated in water when compared to larvae extracted in winter ($p < 0.01$), whereas no significant difference was evident in *C. chiopterus.*

Discussion

A good understanding of the interaction of *Culicoides* with the environment is important in light of the veterinary importance of several species of this genus. A better knowledge on the immature stages, forming the basic of any adult population with the ability to vector pathogens, could be particularly helpful, e.g., for the development of control measures. However, our knowledge on the ecology of the immature stages of *Culicoides* is still very poor. The present study was conducted to provide a more detailed knowledge of the behaviour and reactions towards changes of the environmental conditions, by flooding the larvae and pupae.

The larvae of almost all *Culicoides* species are competent swimmers and show typical rapid swimming flexion when placed in water (Linley, 1986), however, the rates of swimming movements of *Culicoides* larvae vary. In some species they are “so rapid that a larva will appear only as a blur“ (Williams, 1951, p. 174). Sinuous flexions as those described by Linley (1986), were also observed in *C. chiopterus* and *C. dewulfi* in the present study, however, the flexion rate was much lower than that of swimming *C. variipennis* (10.8 ± 0.2 f/s) and did hardly result in forward locomotion in water. Slow flexions were suggested to serve *Culicoides* larvae for the movement in wet particulate or fibrous substrates (Linley, 1986).

Thus, such a behaviour might be of benefit in mediums of higher viscosity, e.g., the more liquid regions of a cowpat. Moreover, the fact that pupae of *C. chiopterus* and *C. dewulfi* are not able to float and the limited tolerance towards longer flooding periods, might be related to the development in cowpats, a habitat, in that the immatures should be rarely confronted with inundation. The "slow head to tail flexion at a rate of less than once per second" described for *C. brevitarsis*, an Australian species developing in cowpats (Cannon and Rye, 1966, p. 8), was also observed in the species investigated in the present study. Cannon and Rye (1966) suggested this behaviour, along with the inability of *C. brevitarsis* to swim or float, to be an adaption to the development in terrestrial habitats. In the present study, the rate of sinuous flexions was twice as fast as in *C. chiopterus* and *C. dewulfi*, possibly indicating an adaption to other kinds of substrates. *C. obsoletus* s.s. is able to develop in a wide range of habitats, e.g., in leaf litter, marshland, tree holes, river edges and dungheaps (Kettle and Lawson, 1952; Murray, 1957; González et al., 2013; Harrup et al., 2013).

Because larvae of some species of *Culicoides* cannot escape from flooded habitats by swimming, such changes of the habitat can affect the survival. The larvae of *C. imicola* survived immersion for at least 13 days while the pupae of this species survives flooding for more than six days, but dies within one day after removal from the water (Nevill, 1976). *C. chiopterus* and *C. dewulfi* in cowpats do not develop to adults after permanent flooding of this habitat for 31 days or alternate flooding for every third day (Lühken et al., 2014). It is not known which life stage was negatively affected by this treatment but in the present study, the pupae were more susceptible to long-term flooding.

In our study, flooding for 24 hours had little or no effect on the development success of larvae in our experiments while flooding for ten days negatively affected the larvae of both species tested, with considerably less larvae of *C. dewulfi* surviving the long term flooding. The flooding of pupae for 24 hours significantly reduced the developmental success of pupae of *C. chiopterus*. In the field, this situation occurs when pastures are temporarily flooded after heavy rains, but drain soon afterwards. Instead, flooding of the pupae for ten days completely prevented successful development in both species.

Culicoides larvae are apneustic and rely on cutaneous respiration. Instead, pupae (of aquatic species) have to float to the water surface to obtain oxygen with prothoracic respiratory horns (Kettle and Lawson, 1952; Kettle, 1977; Nevill et al., 2007). Thus, pupae, that are not able to float, might suffocate more rapidly than the larvae of the same species. The pupae of many *Culicoides* species are able to float when flooded (Dyce and Murray, 1965), but *C. chiopterus* and *C. dewulfi* lack this ability. This inability has also been described for *C. imicola* and *C.*

brevitarsis and, thus, appears to be characteristic for the species of the subgenus *Avaritia* that are expected to develop in moist but not waterlogged substrates (Cannon and Rye, 1966; Nevill, 1967; Nevill et al., 2007). The present study did not allow for the observation of pupae of *C. obsoletus* s.s. or *C. scoticus*. However, Jamnback (1965) mentioned that, except the members of the Obsoletus Group, the pupae of any other species he investigated floated to the water surface.

Pupation in water was evident in submerged larvae of *C. chiopterus* and *C. dewulfi* in the present study. This behaviour was not restricted to overwintering individuals, but also observed in summer larvae. These species continued their development under unfavourable conditions, i.e., when being flooded, even though it decreased the probability of survival. This behaviour is not common in *Culicoides*. Kettle (1962) concluded that pupation does not occur when larvae are flooded. The mature larvae of some *Culicoides* species are known to migrate into drier habitat regions in order to pupate and emerge and some species cannot emerge at all when their pupae are wet (Borkent, 2015). According to Bidlingmeyer (1957), *C. furens* pupates only above the waterline and too much water also hampers the pupation of *C. nubeculosus* (Megahed, 1956). Instead, larvae of *C. imicola* species were observed to pupate in water, however they died soon afterwards (Nevill, 1967).

Steinke et al. (2015) recently demonstrated *C. chiopterus* to be more resistant towards temperatures below zero when compared to *C. dewulfi*. Possibly, the latter species requires more constant conditions in the breeding habitat. Moreover, the two species develop in close coexistence in a spatially confined habitat. Since two species in the same biocenosis cannot occupy the same ecological niche (Hutchinson, 1957), differences in their optimum of environmental conditions are allegeable. However, further research is necessary in order to explain the differences in their susceptibility towards freezing or flooding. More emphasis should be placed on the relations between species-specific behavioural patterns and habitat binding in future studies, as a more detailed knowledge will contribute to our understanding of the ecology immature *Culicoides.*

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Table 1 Development success of larvae and pupae of *C. chiopterus* and *C. dewulfi* after flooding for 24 hours or 10 days.

		Larvae			**Pupae**		
Species	Treatment	n	Emergence rate [%]	ratio ♂:♀	n	Emergence rate [%]	ratio ♂:♀
C. chiopterus	Control	100	93	1.0	50	48	1.0
	flooding: 24 hours	100	91	0.7	100	77	1.2
	flooding: 10 days	100	50	0.7	100	0	
C. dewulfi	Control	100	78	0.7	50	45	2.6
	flooding: 24 hours	100	87	0.9	100	90	1.0
	flooding: 10 days	100	4	0.3	100	0	

11.2 MANUSCRIPTS: CO-AUTHORED

11.2.1 MANUSCRIPT VI

Culicoides biting midge density in relation to the position and substrate temperature in a cattle dung heap

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Parasitology Research 113: 4659-4662

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SHORT COMMUNICATION

Culicoides biting midge density in relation to the position and substrate temperature in a cattle dung heap

Renke Lühken · Ellen Kiel · Sonja Steinke

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Abstract Dung heaps offer warm breeding sites, which might be suitable for a continuing development or even emergence at low air temperatures in winter. Therefore, this study collected substrate samples from the outer surface and core of a cattle dung heap at the beginning of the winter period. We aimed to analyze the density of immature *Culicoides* in relation to substrate position and temperature. We took samples from the outer layer and core of the dung heap at different heights. Floatation was used to extract *Culicoides* larvae from the dung heap samples. In order to rear larvae individually, we separated them in glass tubes. A total of 229 *Culicoides* larvae were extracted from the dung heap samples. Highest densities (99.1 % of all larvae) were recorded for the outer layers of the dung heap but hardly any in the core (0.9 % of all individuals). While the density of larvae was negatively correlated with increasing substrate temperatures, *Culicoides* larvae were found in a temperature range between 7.9 and 38.0 °C (mean 16.6 °C). Extracted larvae were reared to adults. All male individuals were identified as *Culicoides obsoletus* (Meigen), 1818 and all female individuals as *C. obsoletus*/*Culicoides scoticus*. It can be concluded that dung heaps offer temperature conditions, which allow the survival and probably also the development to adults for immature *Culicoides* also under harsh climate conditions in winter.

Keywords Ceratopogonidae · *Culicoides obsoletus* · Dung heap · Temperature

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Short communication

The Bluetongue virus emerged in Europe since the late 1990s, transmitted by several species of biting midges (Diptera: Ceratopogonidae) of the genus *Culicoides* (Mehlhorn et al. 2009; Wilson and Mellor 2009). Moreover, biting midges were also involved in the transmission of the spread of the Schmallenberg virus since 2011 (De Regge et al. 2012; Rasmussen et al. 2012). Both viral diseases resulted in huge economic costs worldwide (Bath 1989; Velthuis et al. 2010; Conraths et al. 2012).

At least in Northern Europe, biting midges need an overwintering strategy to resist harsh climate conditions with low winter temperatures. Although not studied in detail, *Culicoides* species in temperate climate zones are expected to hibernate in a late larval stage (Szadziewsi et al. 1997). Moreover, vector surveillance studies in Northern Europe indicated very low but regular activity of adult *Culicoides* also in winter (Losson et al. 2007; Bauer et al. 2009; Clausen et al. 2009; Hoffmann et al. 2009; Kiel et al. 2009; Mehlhorn et al. 2009). These findings raised a discussion whether or not these individuals represent an overwintering adult population or newly emerged imagines (Losson et al. 2007). The hibernation strategy of biting midges is probably an important factor influencing the overwintering of the Bluetongue virus or Schmallenberg virus (Koenraadt et al. 2014), which is still an unsolved riddle.

Hörbrand and Geier (2009) hypothesized a positive correlation between the number of *Culicoides* in light-trap samples and the distance of the trap to dung heaps. A study carried out on different farm sites in the UK highlighted that cattle dung heaps are colonized by large numbers of overwintering immature biting midges, which were predominantly colonized by overwintering *Culicoides obsoletus* (Harrup et al. 2013). Dung heaps offer warm breeding sites (Husted 1994), which might be suitable for a continuing development or even

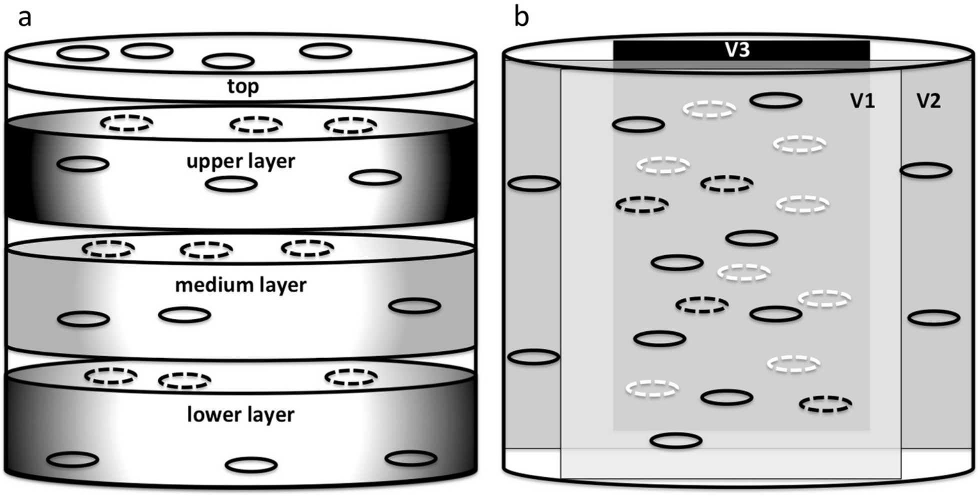

Fig. 1 **a** Schematic diagram of peripheral sampling of dung heap at 26 spots on horizontal layers (top=~3.00–3.30 m, upper layer=~2.00–2.75 m, medium layer=~1.00–1.75 m, lower layer=0–0.75 m). **b** Schematic diagram of core sampling of dung heap at 22 spots, which were randomly chosen on three vertical core layers (V1–V3)

emergence at low air temperatures in winter. Thus, for example, Harrup et al. (2014) hypothesized that the first *Culicoides* emerging in the year originate from dung heaps in which immature stages develop faster.

Depending on the amount of decaying organic material, it seems reasonable that the temperature differs within a dung heap. However, a study focusing on the link between the colonization densities of immature *Culicoides*, the position in a dung heap, and the temperature pattern within a dung heap was not conducted previously. Such knowledge might bring us closer to an understanding of the overwintering ecology of biting midges. We thus evaluated the density of immature *Culicoides* in response to the substrate temperature within a dung heap in Northern Europe at the beginning of the winter period.

This study was performed at the end of October 2008 on a cattle farm in the marshland region of Northwest Germany. The week before, frosty weather had just started and air temperatures at night were below 0 °C. Livestock on this farm comprises about 250 cattle, and the dung heap was located 3–10 m from two cattle stables. The dung had been piled during the last 6 months to a height of approximately 3.30 m and a circumference of 42 m (measured at 1.70 m height). Its outer shape was a broad-based flat pyramid.

At first, we took samples from the outer layer of the dung heap, i.e., surface of the dung heap, at different heights: upper layer ~2.00–2.75 m, medium layer ~1.00–1.75 m, and lower layer 0–0.75 m (Fig. 1). From each layer, we took six samples. Additionally, four samples were taken from the top of the dung heap (top ~3.00–3.30 m). Afterwards, we took samples from the core of the dung heap. To do so, we stepwise trenched the core of the dung heap in three vertical layers (V1–V3; Fig. 1). Each layer was sampled at eight random positions each. Before substrate sampling, we recorded the substrate temperature at every position with a penetration substrate thermometer (PCE-EN 882 Environment Meter, PCE Group, Meschede, Germany).

All samples were taken with a stainless steal grab sampling device. This grab covered a surface area of 15 cm×11 cm and sampled a total volume of 456 ml down to a maximal depth of 7.8 cm. Each sample was stored in 250-ml freezer bag (Ziploc-Tüten, Toppits, Minden, Germany) and transported to the laboratory. Wet weight of each sample was measured with a laboratory scale (Sartorius, LE4202S).

Culicoides larvae were extracted from these samples via a floating method (see Steinke et al. (2014) for methodological details). In order to rear larvae individually, we separated them in 48-ml glass tubes with 8 cm in height and 3 cm in width, which were covered with a cotton gauze. As a reservoir for moisture, the bottom of each tube was grouted with gypsum (approximately 0.5–1.0 cm in height). The gypsum was mixed with some charcoal to prevent massive growth of bacteria and fungi. A total of 229 *Culicoides* larvae were transferred individually on a teaspoon of dung (5–10 g), which had been

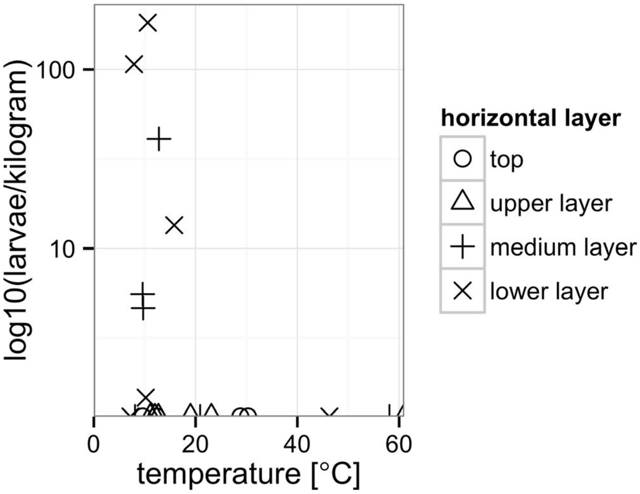

Fig. 2 Immature *Culicoides* densities per kilogram recorded in the outer layer of a dung heap in response to sampling spot temperatures. Further differentiated according to the four horizontal layers: top (N=4), upper layer (N=6), medium layer (N=6), and lower layer (N=6)

carefully floated and sieved previously to exclude invertebrates from the rearing device. For both rearing experiments, the temperature was adjusted to a mean of 21.5±2.0 °C. All samples were regularly wetted with tap water and controlled for adult *Culicoides* every 2–4 days for the following 4 months. Emerged adults were preserved in 70 % ethanol.

Colonization densities (number of *Culicoides* larvae per kilogram substrate) were calculated for each sample. *Culicoides* were identified according to morphological characters (Campbell and Pelham-Clinton 1960). Data analyses were done with the program R (R Core Team 2013) with the package ggplot2 (Wickham 2009) for the graph.

A total of 229 *Culicoides* larvae were extracted from the dung heap samples. Of all *Culicoides* larvae (227 individuals), 99.1 % were found in samples from the outer layer (Fig. 2). Only 2 larvae (0.9 % of all individuals) were extracted from the core material. Most larvae in the outer layer were sampled from the lower layer (204 individuals, 89.1 % of all individuals; Fig. 2), i.e., the bottom layer of the dung heap. Much lower numbers were present in samples from the medium layer (23 larvae, 10.0 % of all individuals), and no larvae were found in the upper layer and the top layer. The number of *Culicoides* larvae per kilogram differed significantly between the horizontal layers (Kruskal-Wallis test, $P<0.05$).

Biting midges were present in samples with temperatures ranging from 7.9 to 38.0 °C (mean 16.6 °C) (Fig. 2). There was a significant negative correlation between the number of individuals per kilogram substrate and the substrate temperature (spearman rank correlation, $P<0.01$, rho=−0.39).

From the 229 larvae, 49 individuals (21.4 %) completed development to adults in the glass tubes. Except for 2 males with damaged hypopygia, all males (28 individuals, 57.1 % of all adults) were identified as *C. obsoletus*, while all females (21 individuals, 42.9 % of the adults) were identified as *C. obsoletus*/*Culicoides scoticus*.

Biting midges are generally expected to colonize the top 2–8 cm of breeding substrates (Mullens and Rodriguez 1992; Blackwell and King 1997). We also recorded highest densities of immature *Culicoides* in the outer substrate layer. Furthermore, larval densities were highest close to the ground, which might indicate a preference for oviposition near to the ground. This conclusion seems to fit to results published by Service (1971), who recorded decreasing numbers of several *Culicoides* species with increasing trap height. However, our result might also be biased by the composition of the dung heap material. Fresh material was generally added on top, but *Culicoides* seem to prefer older, more composted manure (González et al. 2013).

Development of different *Culicoides* species was shown to be temperature dependent (e.g., Veronesi et al. 2009). We found *Culicoides* larvae in a temperature range of 7.9 to 38.0 °C. In our study, development to adults in the laboratory was possible at a temperature around 20 °C. Therefore, it might be concluded that *Culicoides* emergence should also be possible in the field during winter at least at some positions of a dung heap, which offers adequate temperatures for the development of immature biting midges. This might explain the low, but regular, trapping success during winter surveillance (Losson et al. 2007; Bauer et al. 2009; Clausen et al. 2009; Hoffmann et al. 2009; Kiel et al. 2009; Mehlhorn et al. 2009). Adults emerging from dung heaps during winter have the opportunity to stay in a comparatively warm and moist microclimate of a dung heap. If a dung heap is located close to the stable, females should be able to fly to the stable, stay inside after blood meal, and fly back to the dung heap for oviposition without being strongly affected by harsh winter temperatures, e.g., Clausen et al. (2009) caught adult *Culicoides* inside stables during the winter period. But even if a complete life cycle is not possible, due to the different temperatures in the dung heap, a desynchronized emergence of small numbers of *Culicoides* adults is imaginable also during the entire winter period. Furthermore, our results supports the hypotheses by Harrup et al. (2014) that the first *Culicoides* emerging in the year probably originate from warm breeding sites like dung heaps.

Future research regarding the ecology of biting midges should focus on the life-history parameters of *Culicoides* species and should determine temperature thresholds and time of development under different temperature regimes, which was already done for *Culicoides imicola* (Veronesi et al. 2009). This information can be used in mechanistic models to predict *Culicoides* phenology, e.g., probability of winter emergence from dung heaps. Furthermore, it is just as important that future breeding site studies do not omit the winter period, which is not included in most of the studies.

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11.2.2 MANUSCRIPT VII

Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe

R. Lühken, S. Steinke, A. Wittmann, E. Kiel

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Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe

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ABSTRACT

In Northern Europe, dung-breeding *Culicoides* (e.g., *Culicoides chiopterus* (Meigen 1830) and *Culicoides dewulfi* (Goetghebuer 1936)) are considered to be important vectors of the Bluetongue virus and Schmallenberg virus. The interpretation of their distribution is difficult due to the lack of knowledge about their ecology. Previously, soil moisture and especially flooding were identified as important factors that influence the development of several biting-midge species. Therefore, this experimental study addressed the question whether flooding has a negative impact on the development of immature stages of Obsoletus group species. Ten cowpats were collected, and each was divided into four quarters and kept at different moisture regimes in a greenhouse: (1) "dry" (no water added), (2) "control" (regularly moistened), (3) "alternately flooded" and (4) "permanently flooded", to compare *Culicoides* emergence. Flooding had a significant negative impact on the emergence of *Culicoides*. No individuals emerged from the "permanently flooded" treatment and only two individuals were sampled from the "alternately flooded" treatment. In contrast, the total emergence from the non-flooded samples in the "dry" (96 individuals, 38.6% of all *Culicoides*) and "control" (151 individuals, 60.6% of all biting midges) treatments was considerably higher. Biting midges were predominantly identified as *C. dewulfi* (161 individuals, 64.7% of all *Culicoides*) and *C. chiopterus* (63 individuals, 25.3% of all *Culicoides*). There were no significant differences in emergence between the "dry" and "control" treatments. Our results highlight the importance of soil moisture on the distribution of *C. chiopterus* and *C. dewulfi*. Regarding physiological and behavioural adaptations of other *Culicoides* species, we argue that pupae of *C. chiopterus* and *C. dewulfi* are in danger of drowning when breeding sites are flooded as they cannot float. On the contrary, our results indicate that desiccation might not be harmful to these species.

1. Introduction

Several *Culicoides* species are vectors of pathogens [e.g., Bluetongue virus (BTV), Schmallenberg virus (SBV), or African Horse Sickness virus (AHSV)], among which BTV has received the greatest attention in Northern Europe. Bluetongue virus can cause a non-contagious disease of ruminants, resulting in huge economic losses, e.g., the BTV epidemic 2006–2010 led to costs of more than 250,000,000€ in Germany alone (Conraths et al., 2012). Indigenous biting midges of the Obsoletus and Pulicaris group are considered to be the most important vectors (Ander et al., 2012). The recent epidemic caused by SBV

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0304-4017/

again emphasised the importance of biting midges as vectors of pathogens and once again, members of the Obsoletus group were suspected to be the main vectors (De Regge et al., 2012; Rasmussen et al., 2012).

Due to their veterinary importance, many studies have attempted to identify the environmental factors that drive the occurrence of *Culicoides* species (Baylis et al., 1998; Conte et al., 2003). A variety of data types (e.g., temperature, landscape, land cover) have been used to develop species distribution models, however, because of the lack of basic knowledge concerning the ecology of biting midges (e.g., breeding or resting sites), a comprehensive interpretation of the modelling results is often subject to uncertainties. Several studies have demonstrated the need for information on the species to interpret distribution models for *Culicoides*. The interpretation of models for *Culicoides imicola* led to the prediction that areas with an annual rainfall greater than 1000 mm might be unsuitable for *C. imicola* (Wittmann et al., 2001), because the pupae drown when breeding sites are flooded (Nevill, 1967). Another study revealed modelling results that appear contradictory at first sight. Here, a negative impact of cattle density and a positive impact of pasture cover on the species abundance of *Culicoides impunctatus* were found (Purse et al., 2011), however, due to the knowledge of this species' preference for rush pasture cover (Blackwell et al., 1994, 1999), Purse et al. (2011, p. 174) concluded that the correlations "are probably related to the association of this species in the larval stage with rush pasture cover that arises from light grazing and high soil water content".

Larvae of biting midges belonging to the subgenus *Avaritia* are generally expected to have a slow head-to-tail flexion, in contrast to the serpentine swimming movements of other *Culicoides* species. Furthermore, the pupae are not able to float (Cannon and Reye, 1966). This is interpreted as an adaptation to breeding sites with a relative high viscosity (e.g., dung) and might also explain their breeding site preferences in comparison to other *Culicoides* species. Members of the Pulicaris group are tolerant of, or might even prefer, waterlogged breeding sites, because the pupae can float on the water surface (Nevill et al., 2007; EFSA, 2007). Additionally, there was no negative impact of flooding on the eggs or larvae of seven South African *Culicoides* species and only a negative impact on the pupae of *C. imicola*, which drown under waterlogged conditions (Nevill, 1967).

Controlled experiments of environmental (e.g., soil characters, type of vegetation) and management factors (e.g., manure storage) can help to understand the ecological processes that affect *Culicoides* species distribution in and around farms (Scolamacchia et al., 2013). There still is a deficit of experiments to close the broad gaps of knowledge on the ecology of biting midges. Even the breeding ecology of the exhaustively investigated species *C. imicola*, which is known as the main vector of BTV and AHSV in Southern Europe, the Mediterranean region, and Africa (Mellor et al., 2000), is not fully understood and laboratory studies are particularly lacking (Peters et al., 2013). Soil moisture is expected to be an important factor influencing the occurrence of *Culicoides* species, but the connection between soil moisture and *Culicoides* larval development has not yet received sufficient experimental attention (Mellor et al., 2000), although a deeper understanding would help to interpret different patterns of species distribution.

Due to the current state of knowledge on breeding sites of *Culicoides* species in Europe, members of the Obsoletus group are thought to avoid waterlogged habitats (González et al., 2013). It was supposed that the dung-breeding members of the Obsoletus group (*Culicoides chiopterus* and *Culicoides dewulfi*) are able to colonise the driest habitats, which are occupied by immature *Culicoides* (Kettle and Lawson, 1952). However, this was not deduced from experiments on the impact of flooding on immature stages, but from studies on their breeding habitats (Hill, 1947; Zimmer et al., 2008). Therefore, this study aimed to evaluate the impact of flooding on the development of immature *C. chiopterus* and *C. dewulfi* in cowpats.

2. Materials and methods

On 4 April 2013, we selected 10 cowpats on a farm close to the city of Oldenburg (Lower Saxony, Germany). This farm represents a typical dairy farm situated in rural regions in Northern Germany, with a total area of 195 ha and 100 ha grassland, which is used as pasture or meadow. The pasture from which we collected the cowpats is located at a distance of approximately 250 m from the cowshed and is surrounded by forest, a residential area and a small stream. The pasture had not been used for grazing during the winter, i.e., from October until we took the cowpat samples. Therefore, it is reasonable to assume that the cowpats were approximately 5–6 months old.

From the centre of each cowpat, an area of 14 cm × 14 cm was sampled together with approximately 3 cm of the adjacent soil and was transported to a greenhouse. Preliminary studies demonstrated that cowpats can differ strongly in the abundance of *Culicoides*. Therefore, the sampled cowpat areas were divided into four equally sized, quadratic samples (7 cm × 7 cm) and a different treatment was applied to each sample: (1) "dry": no water was added to the samples, (2) "control": each sample was moistened with a pressure spray device every three days (ca. 10 mL tap water per quarter), (3) "alternately flooded": each sample was alternately flooded with tap water (for 24 h, water level ca. 11.5 cm) or not flooded (for 48 h), and (4) "permanently flooded": each sample was permanently flooded with tap water (water level ca. 11.5 cm). Water loss due to evaporation was replaced daily with tap water.

Samples were placed under emergence traps and were covered with a collecting jar. The collecting jars were filled with saturated salt solution to catch and preserve the emerging insects (Fig. 1). The emergence traps of the "alternately flooded" and "permanently flooded" treatments were placed in plastic trays (60 cm × 40 cm × 40 cm, five emergence traps per tray, two trays per treatment). Four small holes (Ø 1 cm) were drilled into the base of the emergence traps and were covered with gauze (mesh size: 125 μm). These holes allowed the filling and draining of the emergence traps with tap water, but the gauze prevented the cowpat material and its coloniser to be washed out.

Collecting jars of the emergence traps were emptied daily. Sampling, refilling and draining were conducted at

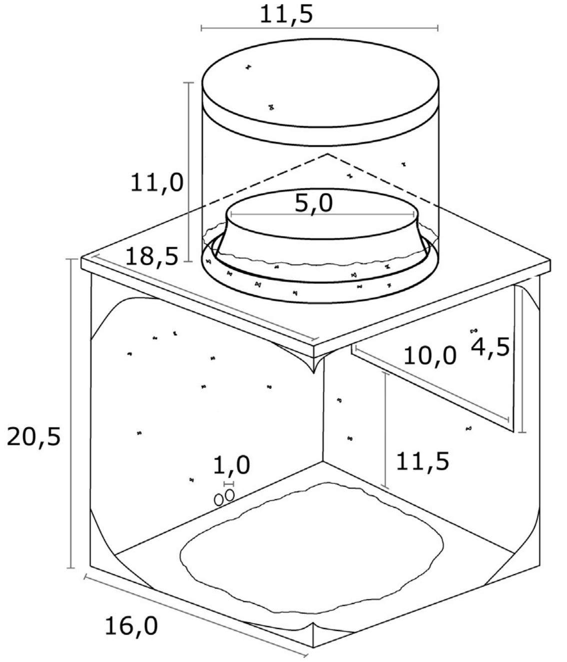

Fig. 1. Schematic drawing of an emergence trap [area = 256 cm^2]. Emerging insects fly towards the light and are trapped in a saturated salt solution. Each emergence trap has two aeration windows covered with gauze (mesh size = 125 μm) to ensure gas exchange and prevent warming within the trap. Indications of size are given in cm. At the bottom of the emergence traps, four small holes (Ø 1 cm) were drilled to facilitate filling and draining and covered with gauze (mesh size = 125 μm) to prevent the cowpat material and its coloniser to be washed out.

the same time (ca. 14–16 h) for all treatments. Oxygen and water temperature in the water were measured daily in the "permanently flooded" treatment and after refilling and before draining in the "alternately flooded" treatment (WTW Oxi 330i, Sensor CellOx 325, Germany). One emergence trap in each treatment was randomly selected to record temperature data (logger: HOBO Pendant® Temperature/Alarm Data Logger 8K, ONSET, Bourne, MA, USA). Another data logger recorded the room temperature. The temperature was measured every 4 h. After 31 days from when the last insect emerged from the cowpats (28 May 2013), the experiment was stopped. The samples were sorted and biting midges were determined to the group level (Obsoletus group, Pulicaris group or other Ceratopogonidae). Males and females of the Obsoletus group were determined to the species level based on morphological characters (Campbell and Pelham-Clinton, 1960). Data analysis was conducted with R (R Development Core Team, 2011), using the package ggplot2 (Wickham, 2009) for graphs.

3. Results

We collected a total of 249 *Culicoides* biting midges belonging exclusively to the Obsoletus group. All except for one cowpat (90%) were colonised by biting midges. The emergence per cowpat showed a high variability (mean = 24.9, max = 123, min = 0). The majority of the 161 individuals (64.7%) were determined as *C. dewulfi* and a further 63 individuals (25.3%) as *C. chiopterus.* Eleven male individuals (4.4%) were identified as *Culicoides scoticus*. Three female individuals (1.2%) were identified as either *Culicoides obsoletus* or *C. scoticus* (Fig. 2), as the morphological differentiation of these two species is difficult. A further 11 male individuals (4.4%) were not determined to species level because their hypopygia were destroyed.

Flooding proved to have a significant impact on the development of *Culicoides* from the samples (Fig. 2). Most *Culicoides* emerged from samples in the "dry" (96 individuals, 38.6%) and "control" (151 individuals, 60.6%) treatments, whereas two individuals (0.8%) emerged from the "alternately flooded" treatment and none from the "permanently flooded" treatment. There were no significant differences between the numbers of *Culicoides* emerging from the "dry" and the "control" treatment (*U*-test, $p > 0.05$; Figs. 2 and 3). All of the three detected *Culicoides* species emerged from the "dry" and "control" treatment, but only *C. chiopterus* emerged from the "alternately flooded" treatment. There were no significant differences in *C. chiopterus* and *C. dewulfi* emergence between the "dry" and "control" treatment (U-tests, $p > 0.05$) (Figs. 2 and 3). Due to the low number of *C. scoticus*, we did not apply a statistical test to compare "dry" and "control" treatments for this species.

The daily mean temperatures in the emergence traps fluctuated between 15 and 20 °C (mean = 18.5 °C, SD = ± 2.2 °C), but were similar between the four treatments and were close to the room temperature. Slightly higher daily mean temperatures occurred in the "permanently flooded" treatment (Fig. 4). Despite high fluctuations in oxygen saturation in the "permanently flooded" treatments, the oxygen saturation differed between the trays, where we placed the emergence traps to achieve flooding. Oxygen saturation in one of the trays dropped significantly from about 100% to approximately 50% during the first few days, but increased during subsequent days and often exceeded 100% (Fig. 5). In the other trays, oxygen saturation decreased much more strongly and did not exceed 80% maximum oxygen saturation until the last day of biting midge emergence (Fig. 5).

4. Discussion

One main outcome of the present study was that *C. chiopterus* and *C. dewulfi* were not able to survive flooding of their breeding substrate. Even alternate flooding of these substrates every 24 h almost completely prevented the emergence of *Culicoides* biting midges. From our experiment, we cannot conclude whether the strong reduction in emergence is caused by a high mortality of larvae, pupae or both. However, the inability of pupae to float is considered to be the most important reason for the breeding-site selection by *Culicoides* species of the subgenus *Avaritia*, habitats of which are described as moist but not waterlogged (Nevill et al., 2007). This agrees with a literature review according to which the members of the Obsoletus group (*C. chiopterus*, *C. dewulfi*, *C. obsoletus* and *C. scoticus*) in the subgenus *Avaritia* (Borkent, 2013) do not occur in waterlogged habitats (González et al., 2013).

The oxygen saturation in the "permanently flooded" treatment strongly decreased at the beginning of the

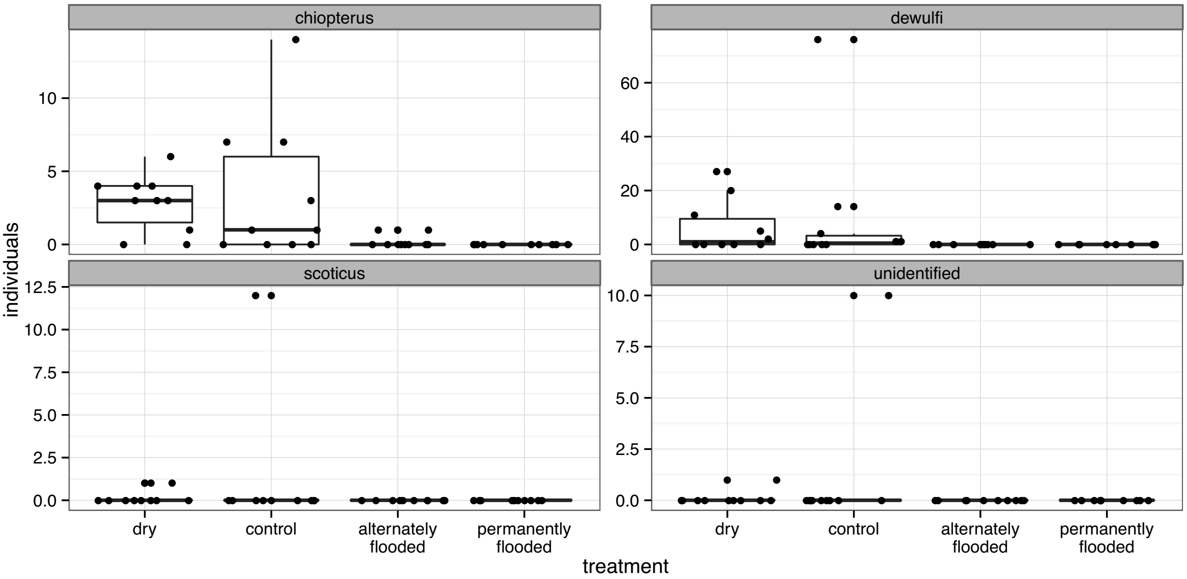

Fig. 2. The number of individuals of *Culicoides* species emerging from samples in the four treatments. A random jitter was added to visualise the raw data.

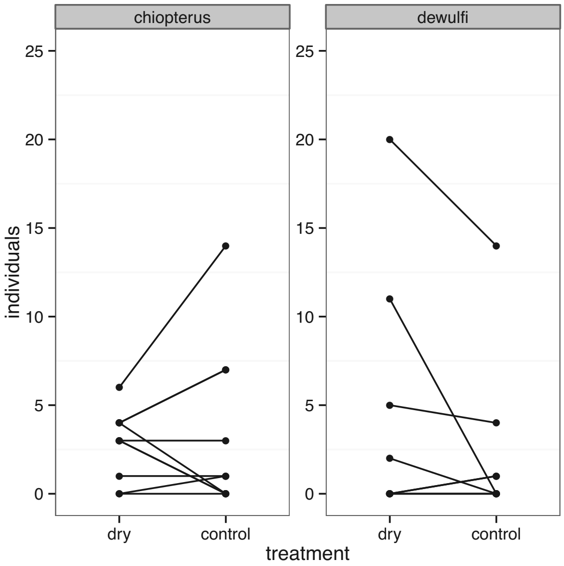

Fig. 3. The number of individuals of *Culicoides chiopterus* and *Culicoides dewulfi* emerging from samples in the "dry" and "control" treatments. The lines connect samples from the same cowpat.

experiment, which might explain the failure of development. However, it can be expected that dung-breeding *Culicoides* biting midges are able to tolerate hypoxic or hypercapnic conditions, because the oxygen content within cowpats can also be low (1–2%) and that of carbon dioxide can be high (25–30%) (Holter, 1991). One counterargument is the vertical distribution of immature stages of *Culicoides*. Eggs are laid on top of the cowpat (Bishop et al., 1996) and larvae/pupae prefer the top layer of breeding substrates (Blackwell and King, 1997; Kettle, 1977; Mullens and Rodriguez, 1992; Zimmer et al., 2008), where the oxygen concentration is higher (Holter, 1991). Additionally, while the cowpats were ageing, the oxygen concentration quickly increased, whereas that of carbon dioxide decreased (Holter, 1991). Thus, dung-breeding *Culicoides* do not have to deal with very low levels of oxygen or very high levels of carbon dioxide during immature development.

Culicoides brevitarsis showed a vertical movement of larvae and pupae towards areas with higher moisture in cattle dung over time (Bishop et al., 1996). Furthermore, experimental studies identified a negative correlation between the water content of dung and the abundance of *C. brevitarsis*, but these results were not supported by field studies (Bishop et al., 2005; Campbell, 1974). The prevalence of this species was not limited by its moisture content (Campbell, 1974), which implies that *C. brevitarsis* is highly adapted to dry habitats. We also did not find any statistically significant differences between cowpats in the "dry" and the "control" treatment (regularly moistened). Therefore, besides the ability of short-range migration towards zones in the cowpat with higher moisture, we assume that coprohphilic *Culicoides* possess physiological adaptations against desiccation (e.g., thickness of the cuticle), which otherwise might be disadvantageous (e.g., inhibiting oxygen uptake) under flooded conditions.

Dung-breeding *Culicoides* (e.g., *C. brevitarsis*) appear to have specific physiological and behavioural adaptations for breeding habitats that are not waterlogged: the pupae are not able to float and the larvae do not show a serpentine swimming characteristic (Cannon and Reye, 1966). Similarly, the eggs and larvae of *C. imicola* can survive flooding, but pupae drown (Nevill, 1967). In contrast, the pupae of aquatic species (e.g., Pulicaris group) are probably able to swim and do not drown when submerged and are therefore able to colonise waterlogged breeding sites (EFSA, 2007). Specific physiological or behavioural adaptations of immature stages might be the key factor in explaining the differences in the breeding site selection between

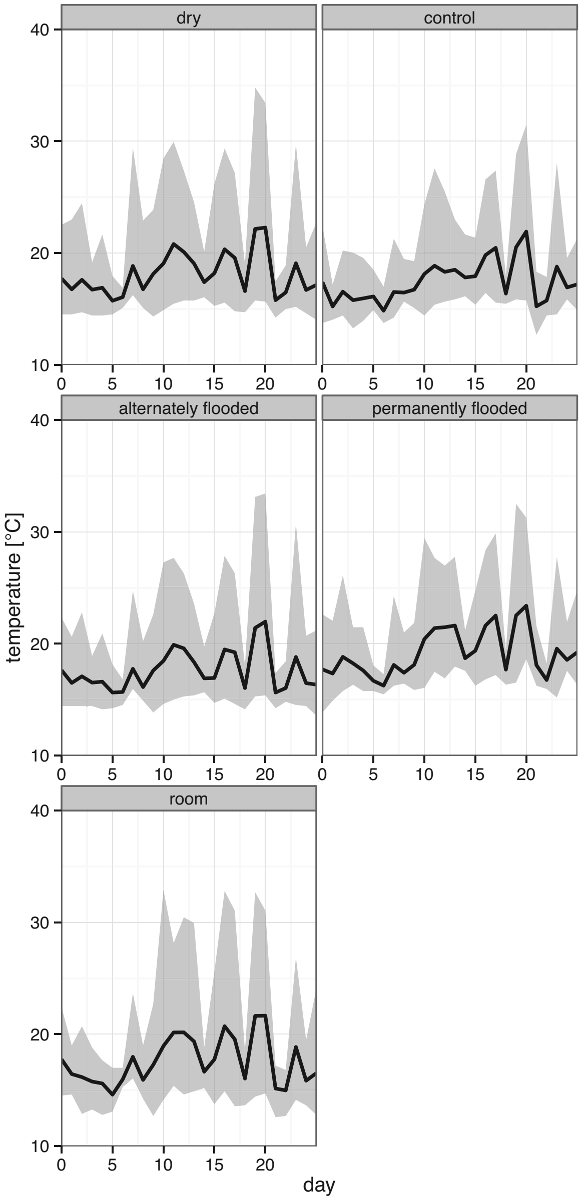

Fig. 4. Daily mean temperature, with daily maximum and minimum values measured with data loggers in the emergence traps of the four treatments, and room temperature. Data from the beginning of the experiment until the day of the last emergence of *Culicoides* biting midges are shown.

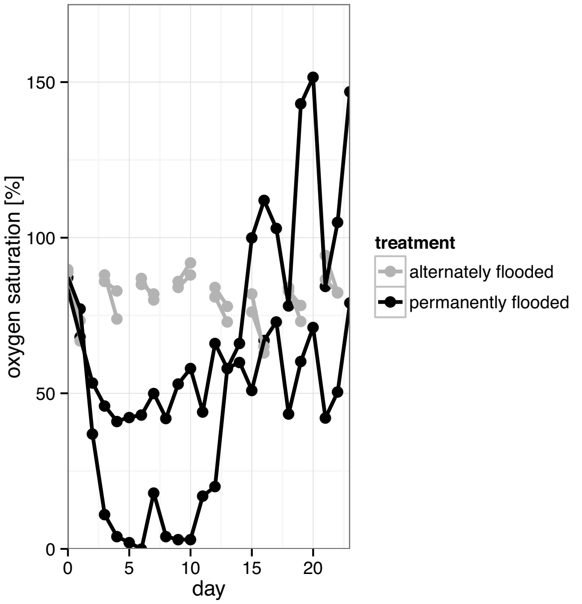

Fig. 5. Oxygen saturation in the water. Daily measurements in both plastic trays of the “permanently flooded” treatment and both plastic trays of the “alternately flooded” treatment, where oxygen saturation was measured immediately after flooding and immediately before draining (24 h period). Data from the beginning of the experiment until the day of the last emergence of *Culicoides* biting midges are shown.

different *Culicoides* species (Nevill et al., 2007), e.g., the females might use volatile chemical cues to identify suitable breeding sites. Floating *Culicoides* pupae use gaseous inclusions within the cephalothorax for buoyancy (Dyce and Murray, 1966). Among the East Australasian *Culicoides* species studied by Dyce and Murray (1966), three types of pupal physiological and behavioural adaptations to different breeding sites were distinguished: pupae of Type A are able to float after flooding, but are not able to submerge again and breed on the margins of still and slow flowing waters, pupae of tree-hole breeding species of Type B can variably float or submerge, and pupae of Type C remain submerged and burrow in the substrate as adaptation for breeding sites in estuarine sands, which are regularly disturbed by flooding or desiccation depending on the tide. Additionally, a Type D was described for *C. imicola*, breeding in moist but not waterlogged soils (Foxi and Delrio, 2010), whose pupae cannot float or burrow and lie on the substratum and drown if flooded (Nevill, 1967). According to the results of this study, *C. chiopterus* and *C. dewulfi* also probably belong to Type D.

In lowland areas of Northern Europe, agricultural intensification is strongly connected to large-scale drainage via ditches (e.g., 300,000 km in the Netherlands) (Verdonschot et al., 2011). These ditches serve to drain rainwater or seepage from groundwater into rivers and lakes, thus resulting in a lower risk of flooding of farmland. Due to the sensitivity of *C. chiopterus* and *C. dewulfi* to flooding, recent agricultural practices might facilitate the populations of both species. Coprophilic *Culicoides* species are suspected to be common species and should be present if cow dung is available (Cannon and Reye, 1966), thus, the species distribution is probably independent from land cover or edaphic conditions. In contrast, both species do not necessarily show an equal distribution (Nielsen et al., 2010) and edaphic variables were found to significantly affect the abundance of both species (Scolamacchia et al., 2013). Our study revealed a clear negative impact of flooding for *C. chiopterus* and *C. dewulfi*. As for *C. imicola*, it is therefore reasonable to expect that both species regularly avoid flooded breeding sites (Foxi and Delrio, 2010). Therefore, soil moisture probably is an important factor for species of the Obsoletus group and should be included in species distribution models, as it was successfully demonstrated for *C. imicola* (Peters et al., 2013).

Acknowledgements

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11.2.3 MANUSCRIPT VIII

Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats

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Parasitology research 113, 1283-1287

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ORIGINAL PAPER

Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats

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Abstract The outbreaks of Bluetongue virus and Schmallenberg virus between 2006 and 2012 highlighted the need for control methods for north-western Palaearctic biting midge species. There is especially a demand for alternative control methods without application of insecticides. Therefore, the objective of this paper was to evaluate the impact of mechanical disturbance as a control method for *Culicoides* biting midges in cowpats. We used a trash rake to destroy the compact structure of cowpats and spread the dung over the ground. We expected the disturbance to cause a faster alteration of physical characteristics and drying of the dung, resulting in lower emergence from disturbed cowpats. Emerging biting midges were collected with emergence traps. The number of emerged *Culicoides* was compared between treated and control cowpats. A total of 12,979 biting midges emerged, all belonging to the Obsoletus group. The majority of the 6,758 male individuals were identified as *Culicoides chiopterus* (Meigen, 1830) (91.4 %), followed by *Culicoides dewulfi* (Goetghebuer, 1936) (6.5 %), and a small number of *Culicoides scoticus* (Downes & Kettle, 1952) (1.2 %). Our results showed no significant differences between the emergence of *Culicoides* (males and females) from disturbed and control cowpats. The lack of differences is discussed in light of the time period chosen for the experiment and the climatic conditions (especially low temperatures) during the study period. The climatic conditions during the study did not favour desiccation effects and therefore did not cause a severe alteration of the dung and the biotic parameters. In conclusion, immature *Culicoides* showed a high tolerance against mechanical disturbance.

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Introduction

Culicoides biting midges are vectors of several pathogens. Currently, two viruses are of major importance in Northern Europe: the Bluetongue virus (BTV) and the 2011 newly identified Schmallenberg virus (SBV) (Hoffmann et al. 2009; Conraths et al. 2013). For several decades, the species *Culicoides imicola* was considered to be the main vector of *Culicoides*-borne diseases in Europe, but the spread of BTV serotype 8 in 2006/2008 across Northern Europe demonstrated the vector competence of indigenous north-western Palaearctic species. Because *C. imicola*, the major vector for BTV in Mediterranean regions, is absent in Northern Europe (Mellor et al. 2000), prior studies about control methods for biting midges in Northern Europe were restricted to nuisance species (e.g. *Culicoides impunctatus*) (Carpenter et al. 2008), which are currently not expected to play a central role in the transmission of BTV and SBV. On the other hand, the rapid succession of BTV and SBV epidemics from 2006 to 2012 highlighted the need for methods to control north-western Palaearctic biting midge species in order to reduce the probability for the transmission of arboviruses. BTV and SBV cause large direct and indirect economic losses (e.g. milk drop, export restrictions): worldwide, the economic damage caused by BTV has been estimated to 3 billion US$ a year (Bath 1989). Because of the significant widespread outbreaks of BTV since this estimation, the actual figure is probably much higher.

Control methods used to reduce both attack rates and transmission of arboviruses by *Culicoides* are predominantly restricted to insecticides, which are used to treat vector resting sites, host animals or screens. Other methods focus on the prevention of vector-host contact (repellents, housing of animals, attractants to lure and kill adult biting midges) (Kettle 1962; Mellor and Wittmann 2002; Carpenter et al. 2008). Recent studies also demonstrated promising results with entomopathogenic fungi or a citronella (natural oil)-based repellent as alternative control agents for adult *Culicoides* (Martínez-de la Puente et al. 2009; Ansari et al. 2010, 2011). As reviewed by Conraths et al. (2012), especially pyrethroids have been successfully used against *Culicoides* spp. and may result in a protection of livestock for several weeks. Many studies positively evaluated pour-on formulations with different pyrethroids (e.g. deltamethrin, permethrin or fenvalerate) to kill *Culicoides* on cattle and sheep (Mehlhorn et al. 2008a, b; Schmahl et al. 2008, 2009a, b). However, most control methods have been proven unsuccessful for the sustainable control of *Culicoides*, e.g. although pour-on formulations were effective even under field conditions (Schmahl et al. 2009c), their application over the whole year is quite disadvantageous (Mehlhorn et al. 2010). In addition, Bauer et al. (2009) used a combination of insecticide-impregnated ear tags, deltamethrin pour-on and insecticide-treated mosquito fences, but they neither found a reduction of *Culicoides* density nor did the methods keep the midges from attacking the livestock. Up to now, there is no proof for a significant reduction of virus transmission by using pour-on formulations (Carpenter et al. 2008). According to the authors, the effects of pour-on formulations are too small to significantly reduce the local midge population. Field studies for other control methods (e.g. entomopathogenic fungi) or a basic knowledge on the ecology of indigenous *Culicoides* species for an effective treatment (e.g. resting habits for an efficient execution of insecticide spraying (Satta et al. 2004)) are still missing.

Potential methods to control *Culicoides* larvae and pupae are insecticides and pathogens, but also habitat modifications or the destruction of breeding sites might be reasonable (Carpenter et al. 2008). The number of studies on breeding sites of potential *Culicoides* vectors is increasing (e.g. González et al. 2013; Harrup et al. 2013). Nevertheless, a comprehensive understanding on the breeding ecology is still missing, thus hampering the effectiveness of a targeted, species-specific application of insecticides. Alternative methods like pathogens as biocontrol agents for immature biting midges were identified (e.g. entomopathogenic fungi; Unkles et al. 2004; Stephen and Kurtböke 2011), but field applications were not conducted yet. Therefore, alternative large-scale vector control methods targeting the breeding sites are predominantly restricted to intensified drainage schemes (Carpenter et al. 2008), an agricultural method which can cause severe ecological problems (e.g. reduced species diversity) (Lu et al. 2009). For that reason, control methods that consider ecological requirements are needed. To our knowledge, up to now, no one has evaluated the effects of manual breeding site disturbance to reduce or even prevent the emergence of biting midges. In Northern Australia, Bishop et al. (2005) found decreasing emergence of biting midges (*Culicoides brevitarsis*) from cowpats, which were naturally disturbed by dung beetles. This result was attributed to the alteration of physical characteristics (e.g. thickness of crust) and faster drying of the dung. Inspired by these studies, we investigated the impact of mechanical disturbance on the emergence of *Culicoides* from cowpats as breeding sites of *Culicoides chiopterus* and *Culicoides dewulfi* (Kettle and Lawson 1952; Dijkstra et al. 2008), both species being considered to be potential vectors of BTV and SBV in Northern Europe (Hoffmann et al. 2009; Rasmussen et al. 2012). This method should simulate the regular activities of farmers, who use harrowing in order to smooth the ground of grassland (e.g. flattening molehills). Pastures generally account for a large area around farms and are intensively frequented by livestock at least during the summer period. Therefore, we assume the disturbance of cowpats as breeding sites for biting midges might be a useful measurement to reduce the vector population and, in this context, also the transmission of arboviruses.

Methods

On April 4, 2011, we randomly selected 46 cowpats on three farms (variable FARM) in Northern Germany. These sites represented typical dairy farms situated in rural regions. The cowpats were taken from a pasture that had not been used for grazing during the previous months, so that they were approximately 5–6 months old. Twenty-three randomly chosen cowpats were mechanically treated with a trash rake, and another 23 cowpats served as a control (variable DISTURBANCE). The disturbance treatment was carried out in a manner that destroyed the compact structure of the cowpat and spread the dung over the ground. At the same date, emergence traps were set up on all disturbed and control cowpats (Fig. 1). The traps ($0.8\ m^2$) covered the entire area of every cowpat. Traps were emptied two times in intervals of 14 days. The samples were sorted in the laboratory, and biting midges were determined to group level (Obsoletus group, Pulicaris group or other Ceratopogonidae). Males of the Obsoletus group were determined to species level based on morphological characteristics of their hypopygia (Campbell and Pelham-Clinton 1960).

Data analyses were done with the program R (R Development Core Team 2010), using the package "ggplot2" (Wickham 2009) for the graphs. Because of overdispersion, data of all emerged individuals of the Obsoletus group, females of the Obsoletus group, males of the Obsoletus group and *C. chiopterus* males was analysed separately with negative binomial generalised linear models through the function glm.nb from the package "MASS" (Venables and Ripley 2002). The variables DISTURBED, FARM, and the interaction DISTURBED:FARM were implemented in the model.

According to recommendations by Zuur et al. (2009), hypothesis testing for significance of the variables and their interaction was conducted using the likelihood ratio test (function lrtest) from the package "lmtest" (Zeileis and Hothorn

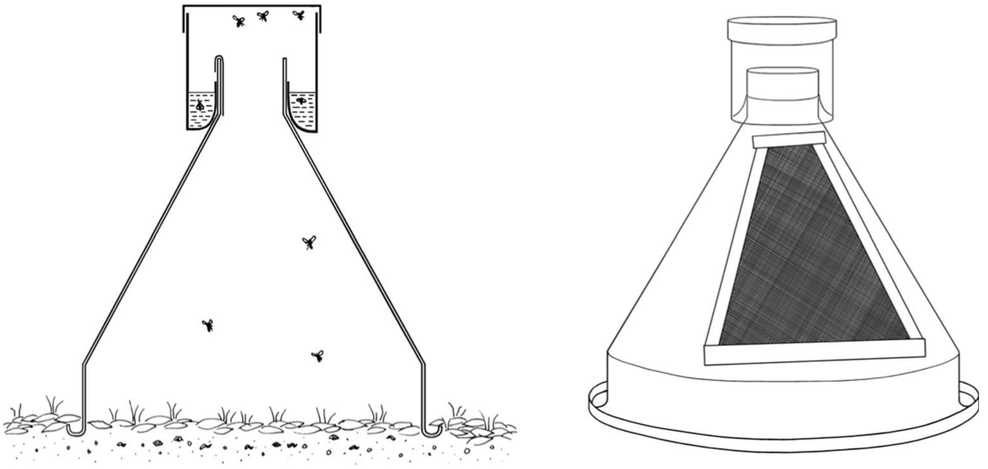

Fig. 1 Schematic drawing of the emergence traps [area=$0.8\ \mathrm{m}^2$, height=32 cm]. Emerging arthropods are attracted by the light falling through the transparent beaker. They were trapped in a conservation liquid (four parts ethanol (96 %), three parts distilled water, two parts glycerine, one part acetic acid and a small drop of washing-up liquid). Each eclector has two aeration windows covered with gauze (mesh size=125 µm), which should ensure gas exchange and prevent the temperatures in the eclectors to become too high. When exposed in the field, the bottom rim was thoroughly covered with soil/substrate in order to prevent the midges to invade from outside (drawings: M. Stöckmann)

2002), dropping each term in turn and comparing the full model with a nested model. The non-significant variables with the lowest impact on the deviance of the model were excluded. The model was then refitted with the remaining terms until all terms were significant.

Results

Beside a large amount of other insects (55,998 individuals), 12,979 biting midges were collected. All biting midges belonged to the Obsoletus group. *Culicoides* emerged from all cowpats with only one exception (97.8 % of all 46 cowpats). Among the 6,758 (52.1 %) males, due to damaged hypopygia, 67 individuals (1.0 %) could not be identified. The majority (6,174 individuals, 91.4 %) were determined as *C. chiopterus*. Only few male *C. dewulfi* (438 individuals, 6.5 %) and *Culicoides scoticus* (79 individuals, 1.2 %) were present. Males of all three species emerged from disturbed and control cowpats. There was a strong variance in the numbers of emerged individuals from the different cowpats (Fig. 2). We found no significant effect of the DISTURBANCE, FARM or the interaction of DISTURBANCE and FARM on the emergence (likelihood ratio tests, $P>0.05$), neither on the total number of emerged individuals belonging to the Obsoletus group, the females or the males of the Obsoletus group, nor on the emergence of *C. chiopterus* males.

Discussion

One option to reduce populations of biting midges is to eliminate or destroy their breeding sites (Carpenter et al. 2008). Firstly, on the local scale, a reduction of potential breeding sites can be achieved through a spatial explicit identification and their removal by an adjustment of the farm management. This includes, e.g., to avoid overflowing cattle troughs, dripping taps or reducing the amount of manure and dung piles directly on the farm. Secondly, on a larger scale, a reduction of biting midge breeding sites is generally undertaken by an intensification of soil drainage (Linley and Davies 1971). Lowering water levels might be suitable to disturb the development of moisture associated *Culicoides* biting midges. In particular, members of the Pulicaris group are expected to be closely associated with high soil wetness and waterbodies (Kettle and Lawson 1952; González et al. 2013), but this does not necessarily match breeding site preferences of the species belonging to the Obsoletus group. While *C. chiopterus* and *C. dewulfi* are expected to breed exclusively in dung (Kettle and Lawson 1952; Dijkstra et al. 2008), *C. scoticus* and *C. obsoletus* s.s. have been sampled from a variety of breeding sites (e.g. dung heaps, manure, dead leaves) (González et al. 2013; Harrup et al. 2013). Thus, an intensification of drainage might not affect the Obsoletus group species. Therefore, in this study, we evaluated an alternative method to control *Culicoides* in cowpats. In temperate regions, *Culicoides* are generally expected to overwinter as larvae (Mellor 1990). In Germany, adult *Culicoides* biting midges were trapped in high numbers after the winter period (Hoffmann et al. 2009). Because the first generation occurs at the end of April or early May, our control measurements at the beginning of May targeted the overwintering immature stages of two members in the Obsoletus group, which probably play an important role in BTV transmission in Germany: *C. chiopterus* and *C. dewulfi* (Mehlhorn et al. 2009). Disturbance affects the physical characteristics of dung (e.g. faster drying) and was

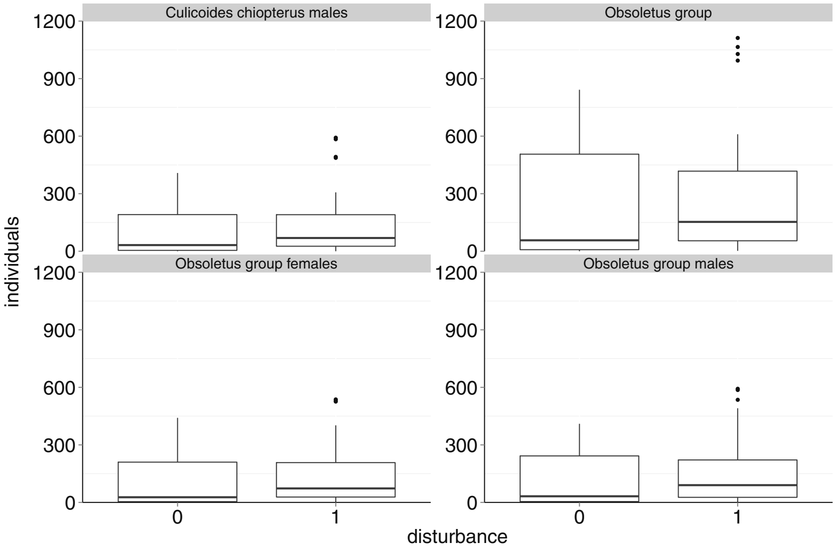

Fig. 2 Number of emerged biting midges from disturbed (disturbance=1, n=23) and control (disturbance=0, n=23) cowpats between April 4, 2011 and May 2, 2011

shown to negatively affect the development of biting midges (Bishop et al. 2005). We assumed the numbers of emerging adults to be significantly reduced when cowpats had been mechanically disturbed. Surprisingly, our results did not confirm this hypothesis, i.e. no significant reduction of emerging *Culicoides* biting midges by disturbance was recorded. For the interpretation of this result, some framework conditions of our study have to be considered. First of all, the time period we chose for the experiment could be important. *Culicoides* larvae overwinter in the third or fourth larval stage (Mellor 1990), which might be relatively tolerant against the kind of mechanical disturbance we applied. Additionally, larvae and pupae breeding in cowpats are generally exposed to harsh environmental parameters during the winter period (e.g. sunlight, temperature, snow or rain) for a comparatively long time. Hence, compared to the natural disturbance during the winter, the mechanical disturbance we applied might have been only of low impact.

Another factor we have to consider when referring to our results is temperature. Differences between our results and the results found by Bishop et al. (2005) for Northern Australia might be due to differences in the climatic conditions. The surface temperatures were much lower during our experiment (−2.9 to 9.3 °C; Germany's National Meteorological Service, www.dwd.de). Therefore, there might not have been enough desiccation to cause a faster alteration of the biotic and abiotic parameters for the immature biting midges.

Although we found no significant differences between the treated and control cowpats, this study may represent a first step towards the development of alternative methods of *Culicoides* control, which are still missing (Mehlhorn et al. 2012). Our results demonstrated huge tolerance of immature biting midges against mechanical disturbance as a single factor. However, further field studies on the impact of mechanical disturbance on *Culicoides* in Europe should be carried out either during dry summer months, in order to analyse the effects of increased desiccation, or should focus on the combination of mechanical disturbance and frost earlier or at the onset of winter. Additionally, laboratory studies under controlled conditions would be advisable to explicitly identify the driving factors, which affect the survival of immature biting midges in their breeding sites. Our study highlighted the general lack of knowledge about the breeding ecology of biting midges. Only few studies focused on the ecological niche of *Culicoides* species, i.e. the variety of factors determining the distribution and development of immature *Culicoides*, in order to identify bottleneck situations suitable for vector control.

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11.2.4 MANUSCRIPT IX

Effects of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *C. dewulfi*

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Short communication

Effects of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *C. dewulfi*

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ABSTRACT

In areas with harsh winters, several studies have recorded low numbers – or even zero – adult *Culicoides* during the winter period followed by a population peak in the subsequent spring. This experimental study determined whether temperature, photoperiod, or the combination thereof affect the development of overwintering immature dung breeding *Culicoides*, resulting in this peak. Temperature had a significant impact on the development period of *Culicoides chiopterus* (Meigen), 1830 and *C. dewulfi* Goetghebuer, 1936. In treatments with constant high temperature, emergence occurred shortly after the beginning of the experiment (mean = day 9). In contrast, no individuals emerged from the other two treatments, as long as the temperatures were below 10 °C. In these treatments, the emergence of *Culicoides* started when the temperature exceeded 20 °C for some days (mean = day 33). There was no significant difference between the two photoperiods (February or April day length; 9 h:15 h [light:dark] vs. 13 h:11 h). Our results highlight the importance of temperature on the spring emergence of *C. chiopterus* and *C. dewulfi*, but the response to the four temperature-photoperiod treatments did not differ between the two species.

1. Introduction

Several *Culicoides* species all over the world are known to be vectors of a variety of pathogens (e.g., bluetongue virus (Mellor et al., 2000), African horse sickness virus (Mellor et al., 2000) and Schmallenberg virus (De Regge et al., 2012; Rasmussen et al., 2012). At least in the temperate climate zone, a recurrent phenological pattern can be observed, with low numbers or a total absence of *Culicoides* adults during the winter followed by a peak of emergence in the spring (e.g., Hoffmann et al., 2009). However, it is unclear what factor or combination of factors (e.g., temperature and photoperiod) regulates the development of the overwintering stages, resulting in this first population peak of the year. Understanding the factors that regulate the development of overwintering, immature *Culicoides* could help to predict the vector phenology and the associated virus epidemiology more precisely, e.g., by adding the factors of temperature and photoperiod into models of vector phenology (Searle et al., 2012).

It has been known for a long time that photoperiod, temperature or the combination of both affects the induction, maintenance and termination of the diapause in insects (e.g., Tauber and Tauber, 1976). This also applies to different *Culicoides* species, whose diapause is controlled by temperature (reviewed by Alekseev et al., 2007). Whereas, to our knowledge, only Isaev (1985) found a combined impact of warm temperature and long photoperiod on the pupation rate of *Culicoides odibilis* Austen, 1921, for other families of the order Diptera (e.g., Chironomidae or Culicidae), there are several examples of species where the photoperiod, alone or in combination with temperature, was found to induce or terminate diapause (reviewed by Alekseev et al., 2007).

Cowpats in northern Germany are predominantly colonized by two *Culicoides* species: *Culicoides chiopterus* (Meigen), 1930 and *C. dewulfi* Goetghebuer, 1936 (Lühken et al., 2015), which are expected to breed exclusively in dung (Kettle and Lawson, 1952), while the differences between the ecological niches are unknown (e.g., Lühken et al., 2015). Furthermore, both species are considered to be potential vectors of important veterinary pathogens (e.g., De Regge et al., 2012). This study aims to compare the emergence patterns of *Culicoides* from cowpats that were experimentally exposed to different temperature-photoperiod treatments. According studies reviewed by Alekseev et al. (2007), which predominantly detected an impact of temperature, it was assumed that high

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Table 1
Temperature-photoperiod treatments exposed to the samples from each cowpat.

Treatment	Temperature	Photoperiod
A	Constant high temperature (20–25 °C)	Long day photoperiod, April day, 13 h:11 h [light:dark]
B	Constant high temperature (20–25 °C)	Short day photoperiod, February day, 9 h:15 h [light:dark]
C	Constant low temperature (<10 °C) at the beginning of the experiment and variable higher temperatures (>15 °C) at the end of the experiment	Long day photoperiod, April day, 13 h:11 h [light:dark]
D	Constant low temperature (<10 °C) at the beginning of the experiment and variable higher temperatures (>15 °C) at the end of the experiment	Short day photoperiod, February day, 9 h:15 h [light:dark]

temperatures, but not photoperiod, triggered the emergence of *Culicoides* from the collected cowpats.

2. Materials and methods

On 3 February 2014, 15 cowpats were selected on a farm close to the city of Oldenburg (Lower Saxony, Germany; latitude 53.1, longitude 8.1). This farm represents a typical dairy farm situated in rural regions in northern Germany, with 195 ha of total land area and 100 ha of grassland used as pasture or meadow. The pasture where the cowpats were collected is located approximately 250 m from the cowshed, surrounded by a small forest, a residential area, and a small stream. As cattle had not grazed on this pasture during the winter, i.e., from October until February, and based on our previous experiences with *Culicoides* breeding sites, it was assumed that the sampled cowpats were between four and six months old. We carefully took into consideration that the cowpats bore the same visual appearance (e.g., same height of grass around the cowpats).

Initially, a small substrate sample (5 cm × 5 cm) was collected from the margins of each of the 15 cowpats, while the rest remained in the field. These rim-samples were analyzed using the Berlese method (Steinke et al., 2014) to evaluate whether or not the cowpats were colonized by *Culicoides* larvae. Four days later (7 February 2014), an area of 14 cm × 14 cm was sampled from the center of each of the nine cowpats that had been found to contain *Culicoides* larvae. These core-samples were taken, along with approximately 3 cm of the soil underneath for moisture-regulation, and then transported to the laboratory. Preliminary studies demonstrated that cowpats could differ strongly in the density of immature *Culicoides*. Therefore, the sampled cowpat areas were divided into four equally sized, quadratic samples (7 cm × 7 cm), which were randomly exposed to a different temperature-photoperiod treatment (Table 1). The two "constant high temperature" treatments, A and B were kept at room temperature between 20 °C and 25 °C, while the two treatments, C and D were kept in a climate chamber. The climate chamber was switched off after 27 days and the temperature in the climate chamber was then influenced by the outside temperature, resulting in "variable higher temperatures (>15 °C) at the end of the experiment". The temperatures studied in this experiment are in the range of soil temperatures that can be measured in the field during February and May.

Samples were placed underneath emergence traps and covered with collecting jars (Steinke et al., 2014). Collecting jars were filled with a saturated salt solution to collect and preserve the emerging insects. The emergence traps allocated to one treatment were placed in one separate lightproof wooden box (length 118.5 cm × width 51.5 cm × depth 50 cm). Each of the wooden boxes was closed with a wooden cover, and a fluorescent lamp (Osram®, Biolux T8 36W, length 121 cm) was included inside the box. Boxes were opened only to empty the collecting jars and moisturize the samples.

The collecting jars of the emergence traps were emptied three days a week (Monday, Wednesday and Friday), resulting in sampling intervals of two and three days. Once a week, the samples were moistened with dechlorinated tap water at room temperature (approximately 20 ml per sample) to prevent drought-induced pupation or emergence. Sampling and moistening was conducted at the same time around noon for all treatments. A data logger (Hobo U23 Pro v2 Data Logger, Bourne, MA, USA) recorded the air temperature in each wooden box at 4 h intervals. Twelve days after the last *Culicoides* emergence was recorded (9 April 2014), the experiment was terminated. Samples were sorted and *Culicoides* were identified to the group level (Obsoletus group, Pulicaris group, other Ceratopogonidae). Males and females of the Obsoletus group were determined to the species level based on morphological characters (Campbell and Pelham-Clinton, 1960; Glukhova, 1989; Delécolle, 1985).

Data analysis was conducted using R (R Development Core Team, 2010). A recently proposed method was applied to analyze the phenology of insects, which was used to identify the timing of the peak of emergence (Searle et al., 2012). The mean number of emerged individuals was calculated for each day of the sampling interval and a three-day moving average of emergence was calculated for each sample and for each species and sex. A separate generalized additive model with a poisson distribution, log link and spline smoothing was fit for each sample using the R package, mgcv (Wood, 2011). Therefore, emergence data were rounded to integer values in order to provide whole the number necessary for the poisson models. The degree of smoothing was selected automatically using generalized cross-validation. The findPeaks function from the quantmod package (Ryan, 2013) was used to identify emergence peaks in the predicted values. Mean and 95% confidence intervals were calculated for the first emergence, the last emergence, the peak of emergence and the total number of emerging individuals for each sex of each species using the summarySEwithin function from the R misc package (Hope, 2013), with the cowpats as subjects and the treatment as a within-subjects variable. Non-overlapping 95% confidence intervals were considered a sign of significant difference. The package, ggplot2 (Wickham, 2009) for graphs and gridExtra (Auguie, 2012) was used for multi-panel graphs.

3. Results

A total of 765 *Culicoides* emerged from cowpats that were exposed to the four temperature-photoperiod treatments. All adults belonged to the Obsoletus group. The majority of the 564 individuals (73.7%) were determined to be *C. chiopterus* and a further 201 individuals (26.3%) were *C. dewulfi*.

Temperature proved to have a significant impact on the development period of both species. In the constant warm environment at temperatures around 22 °C (treatments A and B), the first specimens of *C. chiopterus* and *C. dewulfi* emerged between day 5 and day 18 (mean = day 9) of the experiment, while the last individuals emerged between day 11 and day 31 (mean = day 17) (Fig. 1). The peak of emergence occurred between day 9 and day 20 (mean = day 13) (Fig. 2).

In contrast, no *Culicoides* emerged from the samples in treatments C and D, as long as the temperatures were below 9 °C. Emergence started in these treatments between day 30 and day 39 (mean = day 33), when temperatures rose above 24 °C around day 30, and emergence finally ceased between day 37 and day 60 (mean = day 45) (Fig. 1). The first peak of emergence occurred

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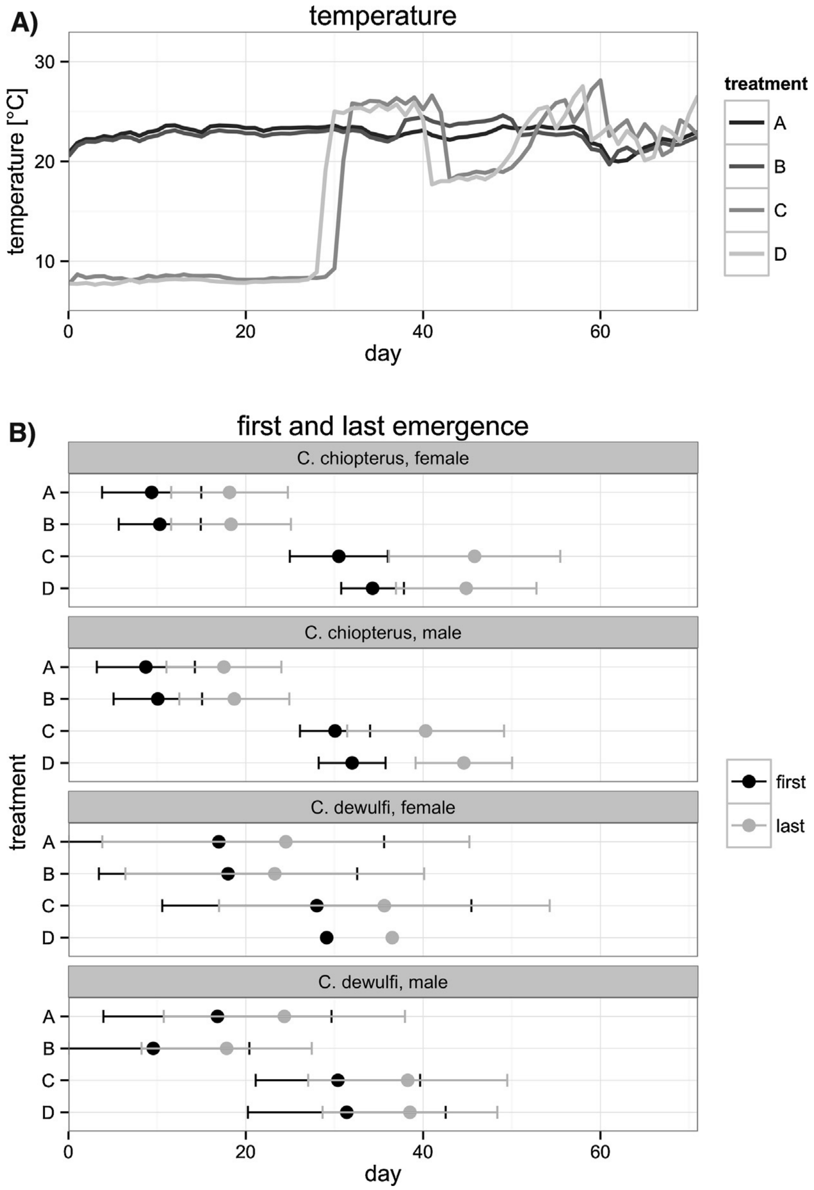

Fig. 1. (A) Mean temperature in the wooden boxes of treatments A–D, (B) mean and 95% confidence intervals of the first and last emergence of *Culicoides* individuals from the cowpats in the four different temperature-photoperiod treatments.

between day 34 and day 42 (mean = day 38) (Fig. 2). In both treatments (C and D), fewer adults emerged when temperatures decreased to 20 °C around day 40, but in a few samples, a second emergence peak for *C. chiopterus* occurred between day 47 and day 59 (mean = day 54) when temperatures increased again.

The two different photoperiods tested in our experiment did not cause different emergence patterns (Figs. 1 and 2).

4. Discussion

At least in the temperate climate zone, there is a recurrent phenological pattern, with low numbers or the absence of *Culicoides* adults during the winter, followed by a population peak in the spring (Hoffmann et al., 2009). However, the explicit factors driving the development of overwintering *Culicoides* resulting in this spring emergence are still unknown.

Our results gave evidence that increasing temperature, rather than changes in the photoperiod, regulates the emergence peak in the spring for *C. chiopterus* and *C. dewulfi*. This missing impact of the photoperiod is in contrast to studies on members of other families of the order Diptera (e.g., Chironomidae or Culicidae). However, it is in concordance with previous studies on *Culicoides* indicating that, for most species, temperature is the key factor triggering spring emergence (reviewed by Alekseev et al., 2007). Immature specimens in the cowpats developed to adults in the laboratory at high temperatures, between 20 °C and 25 °C, independent of the photoperiod. The effect of temperature is supported by various laboratory studies on the life-history parameters of immature *Culicoides*. Allingham (1991) determined a distinct temperature range, with lower and upper temperatures between 26 °C and 33 °C, to limit the development of larvae of *C. brevitarsis* Macfie, 1939. Temperature dependence has also been published for immature *C. imicola* Kieffer, 1913, with a shorter development period at higher temperatures (Veronesi et al., 2009). Moreover, the developmental periods of *C. variipennis* (Coquillett), 1901, *C. arakawae* (Arakawa, 1910), and *C. maculatus* Zilahi-Sebess, 1936 have been explained with different temperature thresholds (Kitaoka, 1982; Vaughan and Turner, 1987).

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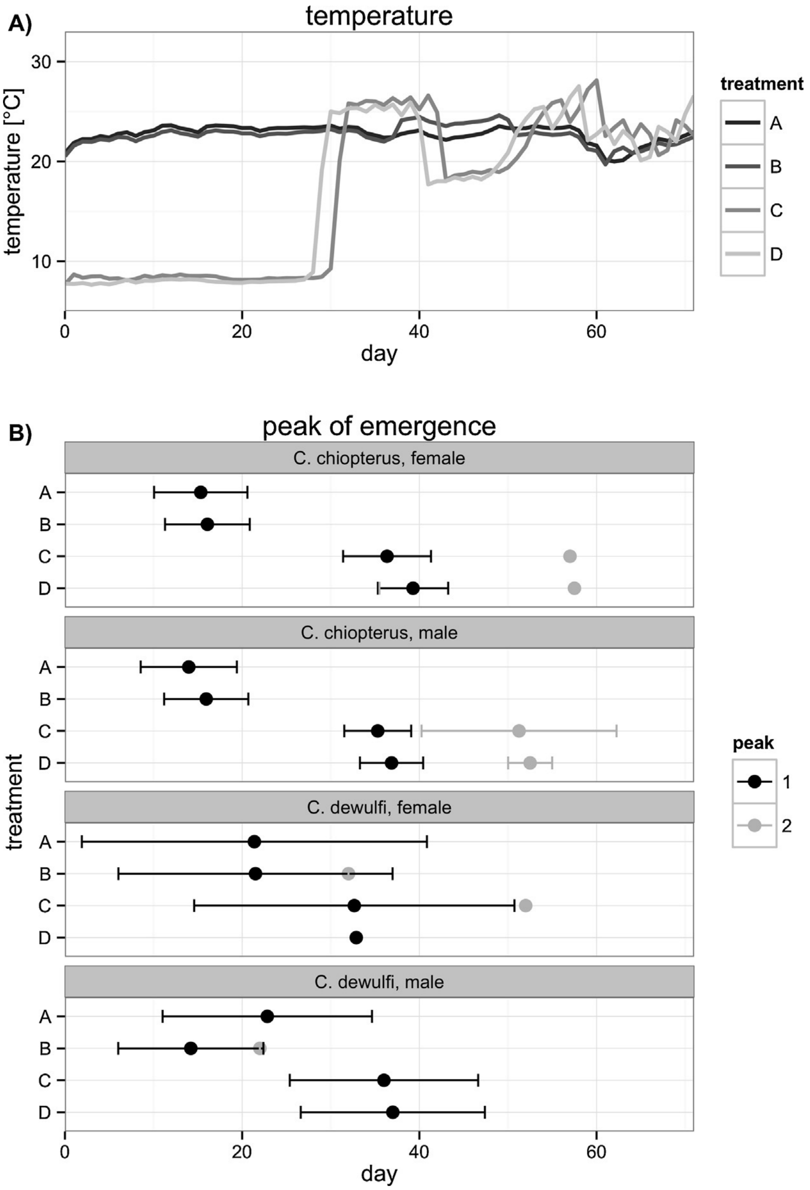

Fig. 2. (A) Mean temperature in the wooden boxes of treatments A–D, (B) mean and 95% confidence intervals of the first and second peak of emergence of *Culicoides* individuals from the cowpats in the four different temperature-photoperiod treatments.

Although our data indicate a clear impact of temperature but no effect of the two different photoperiods studied, further questions remain unanswered, e.g., the diapause mechanism allowing overwintering larvae of *Culicoides* to withstand harsh winter conditions. As in other taxa (Belozerov, 2008), eco-physiological information on overwintering *Culicoides* is lacking. Thus, it is unknown whether the immature *Culicoides* collected in February were in diapause (controlled endogenously) or in quiescence (controlled exogenously). Such detailed information would allow for a more accurate understanding of the *Culicoides* spring phenology. Furthermore, knowledge on the impact of the winter development of immature stages on the vector population is vague. However, this knowledge is essential in order to understand the ecology of the *Culicoides* vectors and associated pathogens. For example, overwintering blackfly larvae (Diptera: Simuliidae) are larger adults resulting in a higher survival rate, dispersal ability and reproduction rate of adults, which in turn could result in a higher vector competence (summarized by de Moor, 1982).

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11.2.5 MANUSCRIPT X

Topsoil conditions correlate with the emergence rates of *Culicoides chiopterus* and *Culicoides dewulfi* (Diptera: Ceratopogonidae) from cowpats

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ORIGINAL PAPER

Topsoil conditions correlate with the emergence rates of *Culicoides chiopterus* and *Culicoides dewulfi* (Diptera: Ceratopogonidae) from cowpats

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Regine Fladung

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Abstract *Culicoides chiopterus* (Meigen), 1830 and *Culicoides dewulfi* Goetghebuer, 1936 (Diptera: Ceratopogonidae) are considered to develop exclusively in dung, but do not necessarily show an equal distribution and abundance on livestock farms in Northern Europe. Recent modelling studies identified soil parameters to explain these differences. The present study addressed the question whether topsoil conditions underneath cowpats correlate with the number of emerging *C. chiopterus* and *C. dewulfi*. We recorded the emergence of biting midges from 24 cowpats over a period of 4 weeks and analysed samples from the topsoil. In agreement with species distribution models based on remote data, our results detected the correlation of soil moisture, organic matter and soil texture with the number of emerging *C. chiopterus* and *C. dewulfi*. With increasing soil moisture, the number of emerging adults increased for both species and the amount of organic matter was positively correlated with the number of emerging *C. chiopterus*. In contrast, soil textures showed conflicting results, i.e. a positive and negative relationship with the same variables. According to our results, soil underneath dung can explain the number of emerging *Culicoides* species. The knowledge of these effects might improve the interpretation of large-scaled distribution models for dung-breeding biting midges.

Keywords Ceratopogonidae · *Culicoides* · Cowpat · Edaphic parameters · Soil wetness

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Introduction

Culicoides chiopterus (Meigen), 1830 and *Culicoides dewulfi* Goetghebuer, 1936 are considered to be vectors of veterinary important pathogens (Mellor et al 2000; Meiswinkel et al 2007; Dijkstra et al 2008), among which the bluetongue virus (BTV) got the highest attention in Central and Northern Europe. BTV can cause non-contagious disease of ruminants, resulting in huge economic losses worldwide, e.g., the BTV epidemic 2006–2010 caused losses of more than 254 million euro in Germany (Conraths et al 2012). The more recent epidemic of the Schmallenberg virus (SBV) also highlighted the importance of biting midges as vectors of pathogens (Elbers et al 2013). Here again, *C. chiopterus* and *C. dewulfi* are suspected to be important vectors (Rasmussen et al 2012; De Regge et al 2012).

C. chiopterus and *C. dewulfi* are expected to breed exclusively in dung (Kettle and Lawson 1952). Therefore, both species were assumed to be commonly present on livestock farms, as it was also proposed for the dung-breeding *Culicoides brevitarsis* (Cannon and Reye 1966). As summarized by Scolamacchia et al. (2013), light traps have been found to underestimate *C. chiopterus* (Carpenter et al 2008; Viennet et al. 2011), but it is generally considered that the trapping results are proportional to the real midge population at the sampling sites. Nevertheless, several studies demonstrated that the distribution and abundance of both species differ (Nielsen et al 2010; Scolamacchia et al 2013). According to the statistical analyses of light trap data, soil parameters of the surrounding environment affected the abundance of these species in opposite ways: the abundance of *C. dewulfi* showed a negative correlation with moisture-retentive soils and a positive correlation with organic content, whereas *C. chiopterus* behaved quite the opposite way (Scolamacchia et al 2013).

The objective of this study was to compare the colonization densities of *Culicoides* biting midges in cowpats with a set of edaphic parameters characterizing the topsoil underneath. A better knowledge of this aspect of *Culicoides* breeding ecology will help to understand the distribution of *Culicoides* species, e.g. interpretation of modelling results using soil information.

Material and methods

On 4 April 2011, we randomly selected 12 cowpats from one pasture on each of two livestock farms close to the city of Oldenburg (Lower Saxony, Germany), which are situated in a distance of 8 km to each other. One of the farms was characterized by relative wet conditions, i.e. marsh land, and the other farm is located on comparatively dry geest land soils. These farms represent typical dairy farms situated in rural regions in Northern Germany. The pastures from which we collected the cowpats were located at a distance of approximately 250 m from the stable. The pastures had not been used for grazing during the winter, i.e. from October until we took the cowpat samples. Therefore, it is reasonable to assume that the cowpats were approximately 5 to 6 months old.

The sampling design aimed at larvae overwintering within the cowpats. Thus, the cowpats were probably only colonized by one developmental stage, which is an advantage for a comparison of cowpat colonization. Emergence traps (see figure in Lühken et al. (2014)) were set up on all of the cowpats we selected for our experiment, which were situated in different situation of soil wetness. The traps of a pyramidal form (area 0.8 m^2, height 32 cm) covered the entire area of every cowpat. When exposed in the field, the bottom rim was thoroughly covered with soil in order to prevent the midges to invade from outside. Traps were emptied two times in intervals of 14 days. The samples were sorted in the laboratory, and biting midges were determined to group level (Obsoletus group, Pulicaris group or other Ceratopogonidae). Males and females of the Obsoletus group were determined to the species level based on morphological characters (Campbell and Pelham-Clinton 1960), whereas female *Culicoides obsoletus* and *Culicoides scoticus* were considered indistinguishable (e.g. Mathieu et al 2012).

On 2 May 2011, which was the last day of the experiment, we took three soil samples (depth=5 cm, area=2 cm) from underneath each cowpat. In order to record soil wetness, these substrates were transported to the laboratory in plastic bags. Fresh weight was measured immediately (Sartorius CP324S, precision 0.001 g) and the dry weight was assessed after the substrate had been dried at 105 °C for 2 days and cooled down to room temperature in the desiccator. Afterwards, the samples were exposed to 400 °C for 4 h and cooled down in the desiccator again in order to measure the ash weight. The difference between fresh and dry weight expresses the water content of the substrate, i.e. soil moisture, while the difference between dry weight and ash weight gives the amount of organic matter. Finally, we analysed the grain size distribution of the samples. Each sample was flushed through a sieve cascade (1000, 500, 200, 63 and 20 μm) with tap water. The finest fraction was collected (<20 μm) in a collection pan. The sieve residues from each mesh size were dried at room temperature and weighted. This resulted in a percentage distribution of six grain size classes: grain size 6 (≥1000 μm, very coarse sand), grain size 5 (≥500 μm, <1000 μm, coarse sand), grain size 4 (≥200 μm, <500 μm, medium sand), grain size 3 (≥63 μm, <200 μm, fine sand), grain size 2 (≥20 μm, <63 μm, silt) and grain size 1 (<20 μm, clay). All results of the three soil samples from underneath the same cowpat were averaged.

Statistical analyses of the biting midge abundance were conducted according to the publication of Goodenough et al. (2012). The authors described a new method of variable selection called "Regression with Empirical Variable Selection" (REVS), which were demonstrated to have a better performance than full, stepwise or all-subsets models. For both species (*C. chiopterus* and *C. dewulfi*), all-subset regressions were conducted. The function uses branch-and-bound techniques to calculate all combinations of variables for each level, while level means the number of variables per model at any one time. The best model with the highest R^2 at each level was selected. From this subset of models, the amount of empirical support was quantified for each variable by the number of models the variable was included in. Finally, models were built according to the empirical support for each variable. The first model only includes the variable with the most empirical support, the second model includes this variable and the variable with the next most empirical support and so on. These models were than compared using the delta AIC (Akaike information criterion) values. Models with a delta AIC>10 were interpreted as not supported, while models with delta AIC<2 were interpreted as strongly supported. Additionally, the commonly used stepwise regression was applied using the step-function in R. The best REVS model and the best stepwise model according to their AIC values, where compared with adjusted R^2, AIC, delta AIC and p values. See Goodenough et al. (2012) for further details on the modelling strategy and the R script.

Leave-one-out cross-validation of the predictive accuracy of each model was applied to estimate the mean square prediction error using the R library boot. To quantify the severity of multi-collinearity, variance inflation factors (VIF) were calculated for each predictor. VIF>10 were interpreted as severe collinearity. Spatial autocorrelation was accessed via variogram plots for the residuals of the linear regression models (Zuur et al 2009). The data structure of the environmental variables was evaluated with Spearman's rank correlation.

Results

We collected a total of 2792 *Culicoides* biting midges belonging exclusively to the Obsoletus group. Six cowpats (25 %) were not colonized by biting midges. The emergence per cowpat varied considerably a high variability (mean=116.3, max=804, min=0). The majority (2397 individuals, 85.9 %) were determined as *C. chiopterus* and a further 366 individuals (13.1 %) as *C. dewulfi*. Twenty-three male individuals (0.8 %) were identified as *C. scoticus*. Three female individuals (0.1 %) were identified as either *C. obsoletus* or *C. scoticus*, as the morphological differentiation of these two species is difficult. Two further females and one male (0.1 %) were not determined to species level because important identification characters were destroyed.

This study confirms the correlation of topsoil conditions with the number of emerging biting midges from cowpats. Although the stepwise and REVS model had a similar delta AIC, adjusted R^2 and p value for *C. chiopterus*, the stepwise model also included several collinear factors (VIF>10) (Table 1). The REVS model for *C. dewulfi* had a better performance compared to the stepwise model. For *C. chiopterus*, 56 % of the variation in the number of emerged adults could be explained and 40 % of the variation for *C. dewulfi*. The variogram plots for the residuals of the linear regression models did not indicate spatial autocorrelation for any of the models.

Generally, stepwise and REVS models included similar sets of variables (Table 2). Both species showed a positive response to increasing soil moisture, which was included in all REVS and stepwise models. *C. chiopterus* showed a negative correlation with organic matter in the REVS and stepwise model; in contrast, *C. dewulfi* was not significantly affected by this variable. In REVS and stepwise models

Table 1 Final Regression with Empirical Variable Selection (REVS) models and stepwise models for the number of emerging *Culicoides chiopterus* and *C. dewulfi* from cowpats

Species/model/parameter	Estimate	SE	*t*	*p*	VIF	AIC	Delta AIC	Adj R^2	*p*	MSE
Culicoides chiopterus										
Best REVS model						234.591	0.000	0.561	0.002	1885.762
Intercept	−192.800	203.622	−0.947	0.357						
Site	379.144	91.968	4.123	0.001	2.785					
Soil moisture	11.275	2.815	4.005	0.001	1.305					
Grain size 2 (≥20 μm, <63 μm)	36.355	11.134	3.265	0.005	2.177					
Organic matter	−15.892	5.688	−2.794	0.012	2.807					
Grain size 6 (≥1000 μm)	84.237	53.156	1.585	0.131	1.647					
Grain size 4 (≥200 μm, <500 μm)	−11.197	4.830	−2.318	0.033	1.958					
Best stepwise model						234.926	0.335	0.565	0.003	2482.687
Intercept	3479.522	927.671	3.751	0.002						
Site	458.657	112.836	4.065	0.001	4.128					
Soil moisture	10.641	2.860	3.721	0.002	1.306					
Organic matter	−11.566	6.686	−1.730	0.103	33.038					
Grain size 6 (≥1000 μm)	93.473	57.957	1.613	0.126	1.466					
Grain size 5 (≥500 μm, <1000 μm)	−49.868	11.781	−4.233	0.001	44.038					
Grain size 4 (≥200 μm, <500 μm)	−37.213	10.949	−3.399	0.004	30.615					
Grain size 3 (≥63 μm, <200 μm)	−82.103	39.897	−2.058	0.056	4.323					
Culicoides dewulfi										
Best REVS model						165.259	0.000	0.407	0.002	1268.449
Intercept	−45.120	16.206	−2.784	0.011						
Soil moisture	2.157	0.643	3.357	0.003	1.051					
Grain size 1 (<20 μm)	79.814	24.742	3.226	0.004	1.051					
Best stepwise model						167.345	2.086	0.375	0.006	1316.142
Intercept	338.213	139.435	2.426	0.025						
Soil moisutre	2.028	0.659	3.079	0.006	1.048					
Grain size 4 (≥200 μm, <500 μm)	−4.441	1.419	−3.130	0.005	2.443					
Grain size 3 (≥63 μm, <200 μm)	−4.000	1.734	−2.307	0.032	2.386					

Estimate estimated regression parameter, *SE* standard error, *t t* value, *p p* value, *VIF* variance inflation factor, *AIC* Akaike information criterion, R^2 coefficient of determination, *MSE* mean square prediction error

Table 2 Spearman's rho rank correlation coefficients and range of the sampled topsoil parameters

	Range (mean, (max, min))	Soil moisture	Organic matter	Grain size 6 (≥1000 μm)	Grain size 5 (≥500 μm, <1000 μm)	Grain size 4 (≥200 μm, <500 μm)	Grain size 3 (≥63 μm, <200 μm)	Grain size 2 (≥20 μm, <63 μm)
Soil moisture	20.3 % (4.9 %, 41.9 %)	*1.00*						
Organic matter	14.4 % (8.0 %, 25.9 %)	*0.42*						
Grain size 6 (≥1000 μm)	1.0 % (0.0 %, 3.2 %)	−0.06	−0.12					
Grain size 5 (≥500 μm, <1000 μm)	2.1 % (0.5 %, 5.4 %)	−0.04	0.17	*0.56*				
Grain size 4 (≥200 μm, <500 μm)	23.8 % (14.5 %, 46.7 %)	0.12	0.2	0.31	*0.58*			
Grain size 3 (≥63 μm, <200 μm)	64.6 % (47.7 %, 74.0 %)	0.03	−0.35	0.14	−0.16	*−0.53*		
Grain size 2 (≥20 μm, <63 μm)	7.6 % (2.0 %, 18.2 %)	−0.11	0.11	*−0.55*	*−0.59*	*−0.76*	−0.06	
Grain size 1 (<20 μm)	0.2 % (0.0 %, 0.9 %)	−0.24	0.08	*−0.59*	*−0.52*	*−0.7*	0.12	*0.83*

Significant correlations ($p<0.05$) are indicated in italic

C. chiopterus emergence was positively correlated with grain size 6 (≥1000 μm) and negatively correlated with grain size 4 (≥200 μm, <500 μm). The REVS model for the species showed a positive correlation with grain size 2 (≥20 μm, <63 μm) and the stepwise model a negative correlation with grain size 3 (≥63 μm, <200 μm) and grain size 5 (≥500 μm, <1000 μm). Furthermore, there was a significant site effect for *C. chiopterus. C. dewulfi* emergence numbers were positively correlated with grain size 1 (<20 μm) in the REVS model and negative with grain size 3 (≥63 μm, <200 μm) and grain size 4 (≥200 μm, <500 μm) in the stepwise model.

According to the explorative data analysis, the small and large grain size variables (grain size 1, 2, 5, 6) only showed a small range of values. Several of the grain size variables were strongly correlated with each other (Table 2). Furthermore, a significant positive correlation was observed between soil moisture and amount of organic matter.

Discussion

This study compared the colonization densities of *Culicoides* biting midges in cowpats with a set of edaphic parameters characterizing the topsoil underneath. The results confirm the correlation of topsoil conditions with the number of emerging biting midges from cowpats. Soil moisture and organic matter as well as soil texture were significant variables in the models for the number of emerging *C. chiopterus* and *C. dewulfi*, whereby these variables explained a significant amount of the emergence variation. The relatively large numbers of not-colonized cowpats match previous studies on dung-breeding *Culicoides* (e.g. Zimmer et al 2014).

The emergence of both species was positively correlated with increasing soil moisture. Furthermore, the model indicated a significant site effect for *C. chiopterus* with higher emergence rates at the farm characterized by relatively wet conditions, i.e. marsh land. Similar results were obtained for closely related species in other studies: in the UK, the genus *Culicoides* and the species *C. obsoletus* was able to colonize a wide range of soil moisture (10.3–63.0 %), but statistical analysis indicated a positive association of the probability of occurrence and soil moisture (Harrup et al 2013). The modelling study by Peters et al. (2013) identified soil moisture as an important environmental variable explaining the distribution of *Culicoides imicola*. Additionally, Scolamacchia et al. (2013) highlighted the availability of moisture as an important factor, for the distribution of *C. chiopterus*, *C. pulicaris* s.s., and *C. obsoletus*/*C. scoticus*. These results match the general considerations regarding the breeding sites of *Culicoides* species of the Obsoletus group, which are considered to be moist but not waterlogged (Nevill et al 2007).

Scolamacchia et al. (2013) reported on a negative correlation between the organic matter of surrounding soils and the abundance of *C. chiopterus* in light traps, but a positive relationship for *C. dewulfi*. Our study supported the modelling results only for *C. chiopterus*, but there was no significant effect for *C. dewulfi*. In general, the amount of organic matter has a positive correlation with the water holding capacity of the soil (reviewed by Saxton and Rawls (2006)). Therefore, it remains ambiguous why in our study the number of emerging *C. chiopterus* and *C. dewulfi* lack a positive response for the amount of organic matter. Moreover, further studies need to analyse why opposing responses occur in these species regarding the effect of organic matter (Scolamacchia et al 2013), even though the number of both species positively correlated with increasing soil moisture.

An increasing proportion of smaller grain sizes correlated with a higher number of emerging *C. dewulfi*, while an increasing proportion of larger grain sizes had a negative effect in the stepwise model. Since the water holding capacity increases with increasing proportion of the small grain size fraction (reviewed by Saxton and Rawls (2006)), our results can be interpreted as an increasing number of emerging *C. dewulfi* with increasing water holding capacity, thus matching a general preference of *Culicoides* for moist conditions. However, our results contradict the modelling of Scolamacchia et al. (2013), whereby *C. dewulfi* prefers less moisture-retentive soils. Furthermore, our models showed conflicting correlations between *C. chiopterus* and the soil texture variables. The abundance of the species seems to be positively affected by an increasing proportion of substrate with larger grain size and at the same time a positive correlation with the proportion of substrate with smaller grain sizes. According to Scolamacchia et al. (2013), *C. chiopterus* positively correlated with moisture-retentive soils, i.e. soils with a higher proportion of smaller grain sizes. We assume that the conflict in our results might be caused by the usage of several correlated variables, which make it difficult to identify the driving variables in our models. Furthermore, the range of values was small in some of the significant grain size parameters and, therefore, might be unimportant by themselves, but might be substitute variables for not involved data.

Although we did not collect environmental parameters from the actual breeding sites of *C. chiopterus* and *C. dewulfi*, i.e. cowpats or the interplay between the environmental conditions in the topsoil and the conditions in the cowpat, our study indicated a significant correlation of topsoil conditions and the number of emerging *C. chiopterus* and *C. dewulfi* from cowpats. This study therefore gave support for recent modelling studies, which demonstrated that the soil moisture (Peters et al 2013) and soil texture (Scolamacchia et al 2013) are important factors explaining the distribution of *Culicoides* species.

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11.2.6 MANUSCRIPT XI

Potenzieller Einfluss von Klimaveränderungen auf Stechmücken und Gnitzen und die Bedeutung lokaler Umweltfaktoren in Deutschland (2014)

R. Lühken, S. Steinke, E. Kiel

Warnsignal Klima: Gefahren für Pflanzen, Tiere und Menschen. 2. Auflage

3.2.17 Potenzieller Einfluss von Klimaveränderungen auf Stechmücken und Gnitzen und die Bedeutung lokaler Umweltfaktoren in Deutschland

Renke Lühken, Sonja Steinke & Ellen Kiel

***Potenzieller Einfluss von Klimaveränderungen auf Stechmücken und Gnitzen und die Bedeutung lokaler Umweltfaktoren in Deutschland** – Stechmücken und Gnitzen standen Jahrzehnte lang nicht im Fokus der deutschen Vektorforschung. Dies änderte sich Anfang des 21. Jahrhunderts, zum einen aufgrund der Einschleppung und Etablierung exotischer, vektorkompetenter Stechmückenarten, zum anderen infolge seuchenhafter Ausbrüche von Infektionskrankheiten, ausgelöst durch zwei von Gnitzen übertragenen Viren: dem Schmallenberg- und dem Blauzungenvirus. Die meisten Folgestudien konzentrieren sich auf die geographische Verbreitung der Vektoren und deren Rolle als Überträger von Krankheitserregern, allerdings fehlen nach wie vor grundlegende Informationen über die Ökologie der Arten. So existiert z.B. für verschiedene Stechmücken- und Gnitzenarten derzeit nur ein unvollständiges Bild über ihre Brutplatzpräferenz. Die meisten Vorhersagen über die Veränderung der geographischen Verbreitung von Vektoren beruhen ausschließlich auf Klimaprojektionen. Abgesehen von den großmaßstäblichen klimatischen Bedingungen sind allerdings auch Veränderungen weiterer wichtige lokaler Umweltparameter, wie z.B. die Austrocknungsfrequenz von Gewässern, zu erwarten. Die Analyse und das Verständnis der Faktoren, welche die lokale Verbreitung und Phänologie steuern, werden es uns erlauben, zuverlässiger Aussagen über zukünftige Entwicklungen zu treffen. Der Artikel beleuchtet den aktuellen Forschungsstand zum Einfluss des Klimawandels auf Stechmücken und Gnitzen und zeigt Forschungslücken auf, die für zuverlässige Vorhersagen geschlossen werden müssen.*

***Potential impact of climate change on mosquitoes and biting midges and the relevance of local environmental factors in Germany** - For several decades research on mosquitoes and biting midges has been neglected in Germany. This situation changed at the beginning of the 21st century due to the introduction and establishment of exotic, vector-competent mosquito species and the spread of two Culicoides-borne diseases: the Bluetongue and the Schmallenberg disease. Most subsequent studies focused on the geographic distribution of the species and their role as vectors of pathogens, but the information about the ecology is still incomplete, e.g., there is a lack of knowledge about the breeding sites of several mosquitoes and biting midges. At present, most predictions on the change of geographic distribution of vectors are based on climatic projections. However, apart from differences in the large-scaled climate, changes of other important local factors are to be expected, e.g. the drought frequency of water bodies. Therefore, studying the environmental variables, which regulate the local distribution and phenology is essential for correct predictions. This paper focuses on the current state of research about the impact of climate change on mosquitoes and biting midges and intends to highlight gaps of knowledge remaining to be closed for reliable predictions.*

Obwohl mehrere Arten von Stechmücken (Diptera, Culicidae; *Abb. 3.2.17-1*) und Gnitzen (Diptera, Ceratopogonidae; *Abb. 3.2.17-2*) potenzielle Vektoren für eine Vielzahl von Krankheitserregern sind, standen sie in Deutschland mehrere Jahrzehnte lang nicht im Fokus der Forschung. Während es bis zur Mitte des 20. Jahrhunderts intensive Studien zur Verbreitung von Stechmücken in Deutschland und insbesondere zur Ökologie der Malariaüberträger der Gattung *Anopheles* gab (Weyer 1938), kam dieser Forschungsbereich mit der Ausrottung der Malaria fast vollständig zum Erliegen (Weyer 1956). Ein noch größeres Forschungsdefizit besteht für die Gnitzen, die in Deutschland niemals im Fokus der Forschung standen, da sie bis vor wenigen Jahren keine nachgewiesene Rolle als Überträger von Krankheitserregern hatten.

Mit dem Ausbruch der Blauzungenkrankheit und der Einschleppung exotischer Stechmückenarten änderte sich dies in Deutschland Anfang des 21. Jahrhunderts schlagartig. Im August 2006 kam es zu den ersten Nachweisen des Blauzungenvirus in Deutschland, Belgien und den Niederlanden (Conraths et al. 2012), wobei hauptsächlich Wiederkäuer an der Blauzungenkrankheit erkranken. Hier kann sie unter anderem zu Fehlgeburten und reduzierter Milchleistung führen. In den folgenden Monaten konnte eine rapide Ausbreitung der Krankheit über ganz Europa beobachtet werden. Alleine in Deutschland verursachte die Blauzungenkrankheit durch Fehlgeburten, Entschädigungszahlungen, Impfungen etc. Kosten von mehr als 250 Millionen Euro (Conraths et al. 2012). Nur kurze Zeit später kam es mit dem 2011 erstmals beschriebenen Schmallenberg Virus (Hoffmann et al. 2012) zu einer weiteren Epidemie. Dieses Virus wird ebenfalls durch

Gnitzen übertragen (Rasmussen et al. 2012). Die Krankheit machte insbesondere durch eine große Anzahl von Früh- oder Totgeburten von Lämmern und Kälbern auf sich aufmerksam und breitete sich ebenfalls rasant in Europa aus (Gubbins et al. 2014).

Auch in Bezug auf die deutsche Stechmückenfauna traten in den letzten Jahren beachtenswerte Veränderungen auf. Unter anderem etablierte sich die exotische Asiatische Buschmücke (*Aedes japonicus*) in weiten Teilen Deutschlands (Werner & Kampen 2013) und es gab regelmäßige Einschleppungen einzelner Individuen der exotischen Asiatischen Tigermücke (*Aedes albopictus*) (Becker et al. 2013). Des Weiteren wurden unterschiedliche Viren in Stechmücken identifiziert (Usutu, Batai, Sindbis) (Jöst et al. 2011a, 2011b, Jöst et al. 2010). Hier fand insbesondere das Usutu Virus starke Beachtung, welches im Jahr 2011 ein Massensterben von Vögeln – vor allem Amseln – in Süddeutschland verursachte (Becker et al. 2012). Eine Infektion mit dem Usutu Virus wurde im darauffolgenden Jahr auch bei einem Menschen in Süddeutschland festgestellt (Allering et al. 2012). Schlussendlich rückten durch jüngst veröffentlichte Arbeiten die parasitischen Nematoden in den Blick. In Deutschland wurde der Hundehautwurm (*Dirofilaria repens*) und der Hundeherzwurm (*Dirofilaria immitis*) in unterschiedlichen Stechmückenarten (Kronefeld et al. 2014) und in Hunden (Sassnau et al. 2013) nachgewiesen. Ein erster autochthoner Fall (*Dirofilaria repens*) beim Menschen trat 2014 auf (Tappe et al. 2014).

Stechmücken und Gnitzen: Aspekte des Klimawandels

In den vergangenen Jahren widmeten sich einige Studien sowohl bei Stechmücken als auch bei Gnitzen der genetischen Struktur von Populationen (Huber et al. 2014b) und der Identifikation verschiedener Arten (Börstler et al. 2014). Weitere Arbeiten analysierten die Vektorkompetenz (Huber et al. 2014a) oder die Möglichkeiten von Kontrollmaßnahmen (z.B. Lühken et al. 2014a). Darüber hinaus konzentrierte sich der überwiegende Teil der Stechmücken- und Gnitzenforschung in Deutschland fast ausschließlich auf eine Beschreibung der geographischen Verbreitung von Vektoren und assoziierter Pathogene.

Es ist davon auszugehen, dass Veränderungen klimatischer Parameter im Zuge des Klimawandels einen Einfluss auf die Verbreitung, Abundanz und Phänologie von Gnitzen und Stechmücken haben werden. Zum Beispiel sind die Reproduktion und Überlebensraten in beiden Gruppen stark von der vorherrschenden Temperatur abhängig (z.B. Gubler et al. 2001). Diese Veränderungen, in der Kombination mit sich ändernden Inkubations- und Entwicklungszeiten von

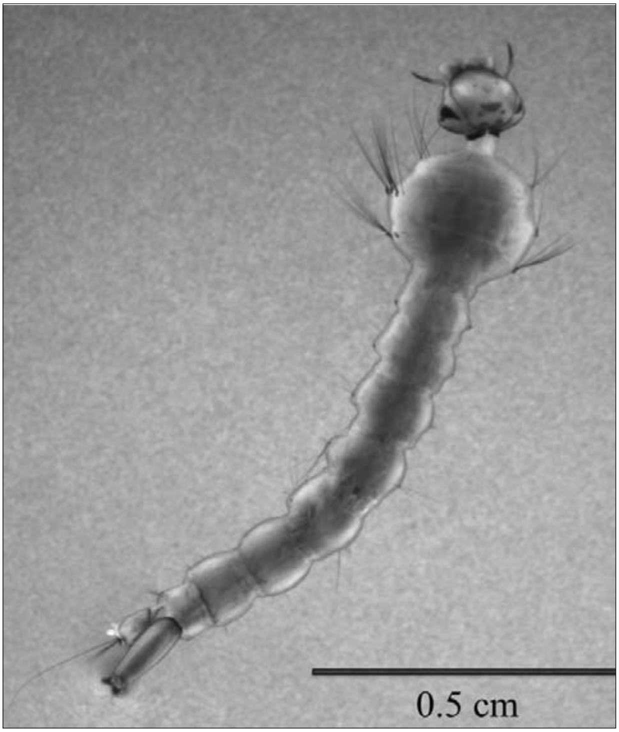

Abb. 3.2.17-1: *Stechmückenlarve.*
Foto: Esther Timmermann (Weitere Fotos in Kap. 3.2.5 - Garms -in diesem Band)

Pathogenen durch steigende Temperaturen, werden aktuell (zusammengefasst von IPPC 2014) und zukünftig (zusammengefasst von Reiter 2001) das Auftreten vektorassoziierter Infektionserkrankungen signifikant beeinflussen. Auf europäischer Ebene gibt es bereits vielfach Ansätze die zukünftige Verbreitung und Phänologie von Vektoren mit Hilfe mathematische Modelle zu analysieren. Modellierungsstudien für Stechmücken hatten dabei oftmals einen Fokus auf die Verbreitung der invasiven Asiatischen Tigermücke. Fischer et al. (2014) haben unterschiedliche Modellierungsansätze im Überblick betrachtet. Im Zuge des Klimawandels und damit einhergehenden wärmeren und feuchteren Überwinterungsbedingungen wird eine zukünftige Ausbreitung der Art nach Norden durch den Klimawandel voraussichtlich begünstigt. Weit weniger weiß man über derartige Zusammenhänge bei einheimischen Stechmückenarten. In Europa ist beispielsweise nur sehr wenig über den Zusammenhang von Klimaparametern und der Populationsdynamik von Vektoren bekannt, was entsprechende Vorhersagen mit Hilfe von Klimaszenarien erschwert (zusammengefasst von Roiz et al. 2014).

Auch für Gnitzen gibt es entsprechende Modellierungsansätze auf Basis von Klimaprojektionen. Die von Afrika ausgehende nördliche Ausbreitung von *Culicoides imicola*, dem wichtigsten Vektors für das Blauzungenvirus in Südeuropa, wird unter anderem mit dem Klimawandel erklärt (Saegerman et al. 2008). In einer anderen Studie haben Acevedo et al. (2010) gezeigt, dass sich die Verbreitung von *Culicoides imicola* in Spanien zukünftig nicht weiter verändert, die Abundanz der Art sich dort, wo sie bereits etabliert ist, jedoch erhöhen wird.

Bedarf an Studien zu lokale Umweltparametern

Derartige Modelle liefern wertvolle Informationen, aber haben natürlich auch Grenzen. Die Datengrundlage basiert für Stechmücken und Gnitzen z.B. überwiegend auf Lichtfallenstudien. Diese Fallen haben zum einen eine unterschiedliche Fangeffizienz für unterschiedliche Arten (Lühken et al. 2014b). Zum anderen erlauben sie keine Rückschlüsse auf realen Populationsgrößen, sondern beleuchten vielfach sehr selektiv, bestimmte Teilgrößen der aktiven Gesamtpopulation (Viennet 2011). Mit CO_2-Fallen werden z.B. überwiegend wirtssuchende Stechmückenweibchen gefangen. Neben diesen grundsätzlichen Problemen mit den Datensätzen für Modellierungen hebt Reiter (2001) hervor, dass der Einfluss von klimatischen Veränderungen zumindest für Stechmücken oft nicht der wichtigste Faktor ist, der die Verbreitung von durch Stechmücken übertragenen Pathogenen bestimmt. So können direkte Einflüsse des Menschen, wie z.B. eine lokale Schaffung von Trinkwasserspeichern, die von Stechmücken besiedelt werden können, einen weit stärken Einfluss auf die Verteilung und Ausbreitung vektorassoziierte Pathogene haben.

Nach wie vor besteht ein großer Wissensbedarf bezüglich der lokalen Faktoren, welche die Verbreitung und Phänologie von Gnitzen und Stechmücken beeinflussen. So existiert insbesondere für verschiedene einheimische Stechmücken- und Gnitzenarten derzeit nur ein unvollständiges Bild über die Brutplatzpräferenz und die biotischen bzw. abiotischen Parameter, welche die Besiedlungs- und Populationsdynamik steuern. Dabei haben viele Studien erfolgreich gezeigt, dass diese Informationen bei Prognosen zur Artverbreitung hilfreich sein können.

So wurden in unterschiedlichen Modellierungsansätzen für die Asiatische Tigermücke Fachkenntnisse über die Autökologie der Art integriert. Die entsprechenden Daten, in diesem Fall zur tolerierten, minimalen Temperatur (Fischer et al. 2014), wurden überwiegend von Fundortdaten abgeleitet, denn es gibt vergleichsweise wenig Laborstudien, die entsprechende Werte liefern. Dennoch lassen sich auf Basis dieser Informationen recht genaue, aktuelle Verbreitungskarten und Prognosen erstellen (Caminade et al. 2012). Gleichzeitig fehlen in Europa noch sehr viele Informationen zu dieser Art. Neben Informationen zur Geschwindigkeit der lokalen, ökologischen Anpassung der Asiatischen Tigermücke, fehlen Kenntnisse über

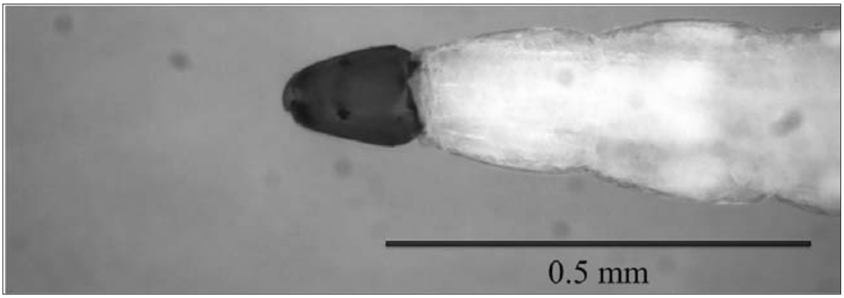

Abb. 3.2.17-2: *Gnitzenlarve. Foto: Sonja Steinke (Siehe auch Kap. 3.2.18 - Mehlhorn - in diesem Band)*

die Anzahl von Individuen, die für eine neue Etablierung in bisher nicht besiedelten Gebieten nötig ist (Fischer et al. 2014). Darüber hinaus gibt es eine Vielzahl weiterer offener Fragen, wie z.B. die Flugdistanz der Art in unterschiedlichen Habitaten oder die Anzahl von Stechmückenindividuen und deren Überlebensfähigkeit, die in Autos oder Lkws aus etablierten Gebieten in nicht etablierte Gebiete transportiert werden. Ein noch größeres Wissensdefizit existiert in Bezug auf die Ökologie der Asiatischen Buschmücke. Unterschiedliche Studien stellten die schnelle Ausbreitung der Art in Deutschland zeitlich und räumlich recht genau dar (Werner & Kampen 2013), gleichzeitig fehlen jedoch Informationen über die biotischen und abiotischen Bedingungen ihrer Brutplätze.

Die meisten europäischen Studien zu Bruthabitaten von Gnitzen auf landwirtschaftlichen Betrieben betrachten die Präsenz oder Absenz in unterschiedlichen Substraten (Kuhfladen, Misthaufen, etc.; *Abb. 3.2.17-3*) González et al. 2013), aber nur sehr wenige Studien analysierten auch biotische und abiotische Faktoren, die einen Einfluss auf die Verbreitung haben. Zimmer et al. (2012) fanden einen negativen Zusammenhang zwischen der Anzahl von *Culicoides obsoletus/Culicoides scoticus* Larven und Puppen und dem Magnesium- und Kalziumgehalt in Silageresten, jedoch eine positive Korrelation mit dem Gehalt von Säuredetergenzienfasern und -lignin. Des Weiteren ermittelte eine Arbeit in England einen positiven Einfluss von Bodenfeuchte und Boden-pH auf das Vorkommen von *Culicoides obsoletus* (Harrup et al. 2013).

Wie bei Stechmücken, zeigten mehrere Studien auch für Gnitzen, dass Informationen über die Autökologie der Arten bei der Interpretation von Modellierungsstudien helfen können. Eine Modellierungsstudie von Purse et al. (2011) hat gezeigt, dass die Abundanz der Art *Culicoides impunctatus* auf den ersten Blick einen widersprüchlichen Zusammenhang zu Umweltparametern zeigte: eine negative Korrelation mit Rinderdichte und einen positive Korrelation mit dem Anteil der als Weide genutzten Fläche. Vorrangehende Brutplatzstudien ermittelten jedoch, dass *C. impunctatus* eine Präferenz für Weiden mit höherer Binsendeckung hat (Blackwell et al. 1999). Somit liegt der Schluss nahe, dass diese Art Weiden präferiert, die aufgrund schwacher Beweidung und hoher Bodenfeuchte einen hohen Binsenanteil aufweisen. Ein weiteres Beispiel ist *Culicoides imicola*, die nach Modellierung von Wittmann et al. (2001) nicht in Gebieten mit Jahresniederschlägen größer 1.000 mm vorkommt. Eine sinnvolle Interpretation wird durch die Kenntnisse der Ökologie der Puppen ermöglicht, die nicht schwimmfähig sind und sterben, wenn die Brutplätze überflutet werden (Nevill 1967).

Fazit

Ein Verständnis und die Analyse der lokalen Faktoren, welche die Verbreitung und Phänologie von Stechmücken und Gnitzen beeinflussen, können genauere Vorhersagen und Interpretationen für zukünftige Entwicklungen im Zuge des Klimawandels ermöglichen. Modellierungsstudien nutzen oftmals eine Vielzahl großmaßstäblicher Daten (Boden, Temperatur etc.), worunter eine Interpretation der genauen ökologischen Zusammenhänge für die jeweiligen Arten leiden kann. Diese Daten können dafür sehr gut für die großflächige Vorhersage der Artverbreitung genutzt und Veränderungen der klimatischen Bedingungen (z.B. IPCC-Prognosen) leicht integriert werden. Für einige Vektoren können jedoch die lokalen Veränderungen durch den

***Abb. 3.2.17-3:** Misthaufen als potenzieller Brutplatz von Gnitzen (Diptera, Ceratopogonidae) mit Emergenzfallen zum Fang emergierender Imagines. Foto: Renke Lühken.*

Abb. 3.2.17-4: *Austrocknendes Stechmückengewässer auf der ostfriesischen Insel Baltrum. Foto: Renke Lühken.*

Klimawandel weit entscheidender sein: z.B. die Trockenphasen und somit die Besiedlungsmöglichkeit von Gewässern für Stechmücken (*Abb. 3.2.17-4*). Ähnliches gilt für die Relevanz der Vektorökologie in demselben Beispiel, bei dem auch entscheidend ist, welche Vektoren solche Trockenphasen ihrer Brutplätze tolerieren können. Spätestens wenn effektive Bekämpfungsmaßnahmen notwendig scheinen, ist eine genaue Kenntnis der Brutplätze und der Ökologie der Vektoren entscheidend (z.B. CARPENTER et al. 2008).

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12. OWN CONTRIBUTIONS TO MANUSCRIPTS

	Manuscript	**Authors**	**Contributions**
First-authored			
I	Assessment of the abundance of *Culicoides chiopterus* and *Culicoides dewulfi* in bovine dung: A comparison of larvae extraction techniques and emergence traps (2014). Veterinary Parasitology 205, 255-262.	S. Steinke R. Lühken E. Kiel	Experimental design: **SS**, EK, RL Data collection: **SS**, RL, EK Data analysis: **SS** Manuscript writing: **SS** Manuscript revision: RL, EK
II	Trapping of *Culicoides* (Diptera: Ceratopogonidae) with emergence traps of different colour and shape. Submitted to Veterinary Parasitology.	S. Steinke R. Lühken F. Kroischke E. Timmermann E. Kiel	Experimental design: **SS**, ET Data collection: FK, **SS** Data analysis: **SS** Manuscript writing: **SS** Manuscript revision: RL, EK
III	Emergence of the *Culicoides obsoletus* group species from farm-associated habitats in Germany (2015). Medical and Veterinary Entomology (in press).	S. Steinke R. Lühken C. Balczun E. Kiel	Experimental design: **SS,** RL, EK Data collection: **SS**, RL, CB Data analysis: **SS** Manuscript writing: **SS** Manuscript revision: RL, CB, EK
IV	Impact of freezing on the emergence of *Culicoides chiopterus* and *Culicoides dewulfi* from bovine dung (2015). Veterinary Parasitology 209, 146-149.	S. Steinke R. Lühken E. Kiel	Experimental design: **SS**, RL, EK Data collection: **SS** Data analysis: **SS** Manuscript writing: **SS** Manuscript revision: RL, EK
V	Experimental studies on the impact of flood events on the development of *Culicodies chiopterus* and *Culicoides dewulfi* with notes on behaviour. Submitted to Veterinary Parasitology.	S. Steinke R. Lühken E. Kiel	Experimental design: **SS** Data collection: **SS** Data analysis: **SS** Manuscript writing: **SS** Manuscript revision: RL, EK
Co-authored			
VI	*Culicoides* biting midge density in relation to the position and substrate temperature in a cattle dung heap (2014). Parasitology Research 113: 4659-4662.	R. Lühken E. Kiel S. Steinke	Experimental design: EK Data collection: EK, RL Data analysis: RL Manuscript writing: RL Manuscript revision: EK, **SS**

VII	Impact of flooding on the immature stages of dung-breeding *Culicoides* in Northern Europe (2014). Veterinary Parasitology 205, 289–294.	R. Lühken S. Steinke A. Wittmann E. Kiel	Experimental design: EK, RL, **SS** Data collection: AW, RL, **SS** Data analysis: RL Manuscript writing: RL Manuscript revision: EK, **SS**
VIII	Impact of mechanical disturbance on the emergence of *Culicoides* from cowpats (2014). Parasitology research 113, 1283-1287.	R. Lühken E. Kiel S. Steinke	Experimental design: EK Data collection: EK, RL Data analysis: RL Manuscript writing: RL Manuscript revision: EK, **SS**
IX	Effects of temperature and photoperiod on the development of overwintering immature *Culicoides chiopterus* and *C. dewulfi* (2015). Veterinary Parasitology 214, 195-199.	R. Lühken S. Steinke N. Hoppe E. Kiel	Experimental design: RL, **SS** Data collection: NH, RL, **SS** Data analysis: RL Manuscript writing: RL Manuscript revision: EK, **SS**
X	Topsoil conditions correlate with the emergence rates of *Culicoides chiopterus* and *Culicoides dewulfi* (Diptera: Ceratopogonidae) from cowpats (2015). Parasitology Reserach 114, 1113-1117.	R. Lühken E. Kiel S. Steinke R. Fladung	Experimental design: EK Data collection: RF, EK, RL Data analysis: RL Manuscript writing: RL Manuscript revision: EK, **SS**
XI	Potenzieller Einfluss von Klimaveränderungen auf Stechmücken und Gnitzen und die Bedeutung lokaler Umweltfaktoren in Deutschland (2014). Warnsignal Klima: Gefahren für Pflanzen, Tiere und Menschen. 2. Auflage.	R. Lühken S. Steinke E. Kiel	Manuscript writing: RL Manuscript revision: EK, **SS**

Abbr.: SS = Sonja Steinke, RL = Renke Lühken, EK = Ellen Kiel, ET = Esther Timmermann, FK = Felix Kroischke, AW = Anna Wittmann, RF = Regine Fladung

13. LIST OF ABBREVATIONS

AIC	Akaikes information criterion
BTV	bluetongue virus
CN-ratio	ratio of carbon to nitrogen
d.f.	degree of freedom
EEA	European Environment Agency
FLI	Friedrich-Löffler-Institut
h	hours
IPCC	Intergovernmental Panel on Climate Change
m	metres
MP3	third segment of maxillary palp
MP4	fourth segment of maxillary palp
n.d.	not determinable
OIE	World Organisation for Animal Health
R^2	conditional coefficient of determination
SD	standard deviation
SE	standard error
ANOVA	analysis of variance
L	litre
°C	Celsius/centigrade

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15. CURRICULUM VITAE

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2006 - 2009	BSc Biological Science, WWU Münster BSc Thesis: Untersuchungen zu physiologischen Reaktionen der infaunalen Bivalve *Scrobicularia plana* auf Veränderungen der Temperatur
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Publications

2016

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2014

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16. ERKLÄRUNGEN GEMÄß § 11 DER PROMOTIONSORDNUNG

Hiermit erkläre ich, gemäß § 11 der Promotionsordnung Fakultät für Mathematik und Naturwissenschaften der Carl von Ossietzky Universität Oldenburg (21.03.2013), dass ich mit dieser Dissertation den Titel Dr. rer. Nat. anstrebe.

Zudem erkläre ich, dass die vorliegende Arbeit selbständig verfasst wurde und nur die angegebenen Hilfsmittel benutzt wurden. Bei der Anfertigung der Dissertation wurden die Leitlinien guter wissenschaftlicher Praxis an der Carl von Ossietzky Universität Oldenburg befolgt. Einzelne Kapitel sind bereits veröffentlicht. Eine entsprechende Publikationsliste (Own contributions) wurde der Dissertation hinzugefügt.

Des Weiteren erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung vorliegt oder vorgelegen hat. Im Zusammenhang mit dem Promotionsvorhaben wurden keine kommerziellen Vermittlungs- oder Beratungsdienste (Promotionsberatung) in Anspruch genommen.

Oldenburg, Februar 2016

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(Sonja Steinke)